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PHYTATE HYDROLYSIS AND FORMATION OF INOSITOL PHOSPHATES IN THE DIGESTIVE TRACT OF BROILERS

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ADF	Acid detergent fibre
BBV	Brush border membrane vesicles
BD_{TW}	Basal diet containing microwave treated wheat
BD_{UTW}	Basal diet containing untreated wheat
BD-	Basal diet without monocalcium phosphate
BD+	Basal diet with monocalcium phosphate
Ca	Calcium
DM	Dry matter
Enz	Enzyme supplementation
GfE	Gesellschaft für Ernährungsphysiologie
HPIC	High-Performance Ion Chromatography
InsPs	Inositol phosphate isomers
$InsP_6$	*Myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)
IUPAC-IUB	International Union of Pure and Applied Chemistry and the International Union and the International Union of Biochemistry
LOQ	Limit of quantification
MCP	Monocalcium phosphate
MCP_h	Monocalcium phosphate monohydrate
MSP_a	Anhydrous monosodium phosphate
Mwt	Microwave treatment
n.d.	Not detectable
NRC	National Research Council
NSP	Non-starch-polysaccharide
P	Phosphorus
Phy	*E. coli*-derived phytase Quantum Blue® or Quantum™ Blue

P_i	Inorganic phosphate
SEM	Standard error of the means
Ti	Titanium
TM	Trockenmasse
tP	Total phosphorus
WPSA	World's Poultry Sciene Association
Xyl	Econase® XT 25

CHAPTER 1

INTRODUCTION AND LITERATURE OVERVIEW

1 INTRODUCTION AND LITERATURE OVERVIEW

1.1 INTRODUCTION

One of the greatest challenges for the society presently is to increase the volume of biomass produced while minimizing the ecological footprint. In this regard, "bioeconomy" is a "new paradigm for bioscience and policy" (Hirsch, 2009) and will represent the key tool to face this challenge. Bio-based economy includes the shift to a sustainable livestock production and responsible handling of natural resources. This change may only be achieved by the interactive use of knowledge and innovation from different sectors, such as agricultural, biological, life and technological sciences. Hence, bioeconomy is also addressed by research in the field of animal nutrition. In terms of animal nutrition research, an important bioeconomical aspect is the development of strategies which result in decreased dependence on limited resources in addition to an efficient use of nutrients contained in plant feed components.

Phosphorus (P) represents a finite resource with major impact in animal nutrition worldwide. P is an essential nutrient for skeletal growth and integrity, serves as a component of phospholipids, nucleic acids and adenosine triphosphate and plays a role in biochemical pathways, fat, carbohydrate, mineral and energy metabolism. Dietary P concentration needs to cover the animals' P requirement, which varies widely and depends on animal factors, such as species, age, physiological state and targeted level of performance (WPSA, 2013). To be utilized, P needs to occur as orthophosphate in the lumen of the small intestine because this is the binding form of P mainly absorbed. Hence, the required dietary P concentration also depends on the contribution of different binding forms and their potential to be dephosphorylated in the digestive tract.

Phytate, the major binding form of P in plant seeds and feedstuffs obtained thereof, can be readily hydrolysed in the rumen due to the presence of microbiota-associated phytases (Yanke et al., 1998). For non-ruminants, the capability to use phytate-bound P has been controversially discussed. Avian species and pigs have long been assumed to be inefficient in utilization of phytate-bound P, due to insufficient endogenous mucosal phytase activity in the intestine (Nelson, 1976). However, more recent studies showed that broilers have a high potential to hydrolyse phytate (Tamim and Angel, 2003; Leytem et al., 2008). But different nutritional factors can affect phytate hydrolysis in broilers, such as intrinsic plant or added microbial phytase, mineral P and Ca and other factors that can affect accessibility or solubility

of phytate. However, the separate and interactive effects of different nutritional factors on the degradation of phytate in the digestive tract are largely not understood.

Theoretically, the total P (tP) concentration of plant-sourced feed ingredients could be adequate to cover the P requirement of poultry. Nevertheless, as a consequence of the variable availability of phytate-bound P, P from mineral sources is often added to poultry diets to meet the birds' requirement. Implementation of a safety margin leads to diets with a much higher tP content than that needed by the bird (Angel et al., 2002b). Consequently, in areas of intensive animal production manure P concentrations can exceed the requirement of the crops grown on the fields (Sharpley, 1999). This can contribute to P accumulation in soil, leaching and surface runoffs of P and its entry into surface and ground water, and can cause eutrophication and thus increased growth of algae and aquatic weeds (Singh, 2008). Hence, a limited resource disappears from the nutrient cycle and can become an environmental pollutant. Policy reacted in several regions by implementing legislation that limits the use of litter application to soil (based partly on soil and litter P content) (Angel et al., 2002a). In Germany, farmers have to keep records and calculate annual P balances, which must not exceed a surplus of 20 kg P_2O_5 per ha (Düngeverordnung, 2007). To reduce the use of mineral P in poultry feed and P in excreta without compromising the bird's health a maximal utilization of phytate-bound P is necessary.

Therefore, improved understanding of phytate degradation in the digestive tract of broilers and the influence of nutritional factors is needed to find, in a second step, solutions to maximize availability of phytate-bound P.

The following sections of this chapter are intended to explain the theoretical framework required to understand the action of different phytases in the digestive tract and the breakdown processes of phytate.

1.2 INOSITOL PHOSPHATES – TERMINOLOGY, STRUCTURE AND NOMENCLATURE

Terminology in regard to inositol phosphate isomers (InsPs) is not uniform in the literature. To avoid confusion in data interpretation, the next subchapters will clarify terms, structures and nomenclature. Isomeric assignments will be explained in detail because positional InsPs so far were not considered in poultry nutrition studies.

Terminology

By definition phytate is any salt of phytic acid. Phytic acid is strictly designed as *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$). In plants and under physiological conditions, phytic acid occurs in its anionic or salt form, not in the free acid form. In literature, "phytin" is often synonymously used with phytate or phytic acid. Shears and Turner (2007) reviewed that this term was originally introduced to describe insoluble Ca/Mg phytate deposits in the globoids of plant seeds. As phytate in the seeds of many species is now known to consist predominantly of Mg/K salts, the term is largely obsolete (Shears and Turner, 2007).

In feed tables, the reported "phytate-P" contents were often determined according to Haug and Lantzsch (1983) or Harland and Oberleas (1986) (De Boever et al., 1994; Eeckhout and De Paepe. 1994; Viveros et al., 2000; Steiner et al., 2007). These non-specific methods do not separate $InsP_6$ and less phosphorylated InsPs (lower InsPs). For samples which contain substantial amounts of lower InsPs, such as processed feed or digesta samples, these methods are inadequate because of an overestimation of the $InsP_6$ content (Konietzny et al., 2006). More recently, high performance liquid chromatography methods were introduced which allow the more precise determination of $InsP_6$ and lower InsPs. To compare results on a common basis in future, it was recommended to represent the $InsP_6$ contents in feed tables based on specific standardized methods for determination of $InsP_6$ and lower InsPs (Schlemmer et al., 2009).

Structure and nomenclature

$InsP_6$ carries six phosphate groups, each esterified with one hydroxyl group of the cyclohexanehexol *myo*-inositol. The abbreviation "Ins" is the IUPAC-IUB-approved (International Union of Pure and Applied Chemistry and the International Union of Biochemistry) abbreviation for *myo*-inositol (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1973). *Myo*-inositol is one out of nine possible configurations of inositol (other stereoisomers are e.g. *scyllo*- or *chiro*-inositol) and the most frequently and ubiquitously occurring isomer in animals, plants and microorganisms. The prefix "*myo*" refers to the conformation of the hydroxyl groups on the inositol ring (Bohn et al., 2008). The most energetically stable conformation of *myo*-inositol is the chair conformation. In this conformation it has one axial (set as position number 2) and five equatorial hydroxyl groups. Thus, there is a plane of symmetry through its 2- and 5-carbons. There are two possible counting methods for numbering the inositol-ring, D- and L-numbering. To avoid confusion,

IUPAC suggested to use Bernie Agranoff's turtle (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1973) (Figure 1).

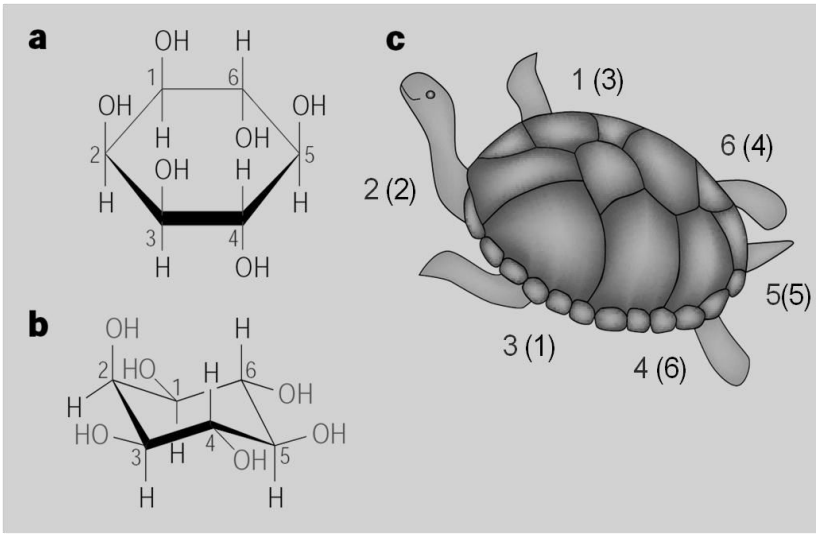

FIGURE 1. Adapted and modified from Irvine and Schell (2001). *Myo*-inositol as Haworth projection (a) and chair structure (b). Agranoff's turtle (c) shows the D-numbering and L-numbering (in brackets) of *myo*-inositol

Agranoff (1978) suggested *myo*-inositol to resemble a turtle. The raised head represents the axial 2-hydroxyl group, limbs and tail represent the five equatorial hydroxyl-groups. Using the D-numbering system means the turtle is right-flippered, so its number 1 flipper is the front right flipper (Figure 1) (Irvine and Schell, 2001). As the head is the 2-hydroxyl group the numbering of the remaining carbons proceeds in the anticlockwise direction (from the plan view of the turtle). L-numbering means the turtle is left-flippered (left front flipper is number 1) and numbering continues clockwise (from the plan view of the turtle). Some confusion may occur in respect to enantiomeric forms of InsPs. Regarding e.g. D-Ins(2,3,4,5,6)P_5 and L-Ins(1,2,4,5,6)P_5, both are dephosphorylated at the right front flipper and thus equivalent. In contrast, D-Ins(2,3,4,5,6)P_5 (equivalent to L-Ins(1,2,4,5,6)P_5) and D-Ins(1,2,4,5,6)P_5 (equivalent to L-Ins(2,3,4,5,6)P_5) are enantiomers, the first being dephosphorylated at the right and the latter at the left front flipper. Enantiomers can be converted to one another by reflection in the mirror plane, but differ in characteristics and physiological functions. To prevent any misconception, IUPAC suggested the term "Ins" to be taken to mean *myo*-inositol with the numbering of the D-configuration unless the prefix L- is explicitly added (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1973). Using high-performance ion

chromatography (HPIC), as done in the present work, InsP enantiomers co-elute. Therefore, in the present thesis "Ins" refers to the *myo*-inositol ring with unknown (D-/L-) configuration.

1.3 PHYTATE

Occurrence in plant seeds

Phytate is the major storage form of P and *myo*-inositol in mature plant seeds, where it accumulates during the ripening process. Detailed information on the content, distribution and occurrence of $InsP_6$ in different feed ingredients were reviewed by Oberleas and Harland, (1981), Reddy et al. (1982), Eeckhout and De Paepe (1994), Ravindran et al. (1994), Angel et al. (2002b) and Bohn et al. (2008). In brief, phytate-P represents between 60% (soybean meal) and 80% (rice bran) of mature seed tP (Ravindran et al., 1994). Less than 10% of tP in dormant seeds is P from InsPs of lower degree of phosphorylation. Thus, phytate is the primary organic P source in pigs and poultry feed, because their diets are mainly based on cereal grains, oilseed crops and their by-products after oil-extraction. Practical poultry diets contain approximately 1% $InsP_6$ by weight which corresponds to 0.28% $InsP_6$-P. This amount varies depending on $InsP_6$ concentration of the respective feed ingredients, which can depend on the prevailing growing conditions, stage of maturity, genetic factors and processing conditions. Eeckhout and De Paepe (1994) reported the following ranges for $InsP_6$-P concentrations: maize, 0.16-0.26% (mean: 0.19%); wheat, 0.19-0.27% (mean: 0.22%); barley, 0.20-0.24% (mean: 0.22%); soybean (extracted), 0.28-0.40%.

The localization of phytate within the kernel also varies between plant species. Generally, $InsP_6$ and the co-precipitated cations are stored in protein storage vacuoles within a membrane-bound compartment, the so called globoid, in the seed (Jiang et al., 2001). It was shown that low-phytate mutants of different cereals had smaller globoids than the corresponding wild type (Liu et al., 2004; Ockenden et al., 2004; Lin et al., 2005). This was seen as the size of phytate globoids to depend on the phytate amount in the grain (Bohn et al., 2008). In maize, approximately 90% of phytate is localized in the germ (embryo and scutellum), whereas the major proportion of phytate (>80%) in wheat, rice or barley is present in the aleurone layer and outer brans (O'Dell et al., 1972). In most oilseeds and grain legumes, phytate is associated with protein and distributed throughout the kernel (Sebastian et al., 1998). Phytate in soybean seeds is unique, because it appears to have no specific localization (Sebastian et al., 1998). In rapeseed, phytate is detected in globoid crystals and remains associated with the denaturated protein in processed rapeseed meal (Yiu et al., 1983).

Localization, surrounding structures and binding form of phytate may be relevant for accessibility, susceptibility and thus degradation in the digestive tract and may contribute to its antinutritive character and environmental impact.

Antinutritive effects

The antinutritive character of $InsP_6$ is primarily caused by the potential to complex positively charged molecules and nutrients, and formation of insoluble salts. It was reviewed that the chelating potential of $InsP_6$ is determined by its negatively charged character (Angel et al., 2002a,b). In brief, the twelve replaceable protons in the $InsP_6$ molecule differ in pK in the range of 1.5-11.0 (Angel et al., 2002b). At the physiological pH values in the broilers' digestive tract phytate carries at least 6 negative charges. The number of negative charges, and thus the potential to bind cations, increases with increasing pH, when digesta moves distally along the broilers' digestive tract (Angel et al., 2002a,b). At higher pH values $InsP_6$ strongly binds and complexes with Ca or other cations (Angel et al., 2002a,b), which can happen in the small intestine. It is commonly presumed that a pH of 5 or higher is pivotal to Ca-phytate precipitation (Selle et al., 2009). Most phytates, especially chelates with divalent mineral cations, are insoluble at high pH values and precipitate. Chelates with monovalent mineral cations such as Na and K are soluble over the full pH spectrum (Selle et al., 2000). Complexes basically cannot be absorbed and hydrolysis of phytate by phytases occurs when phytate is in solution Precipitation causes reduced availability of $InsP_6$-P and chelated nutrients. Degradation of $InsP_6$ down to less phosphorylated derivatives is conducive for solubility (Xu et al., 1992; Sandberg et al., 1999). Solubility of phytate also depends on the complex stability and the mineral to $InsP_6$ molar ratio (Angel et al., 2002a,b). Ca^{2+} shows, among different cations, the lowest affinity for chelating with $InsP_6$, followed by Fe^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} (Vohra et al., 1965). However, Ca has the highest practical impact on $InsP_6$ availability as it is present in highest concentration compared to other metal ions in the diet (Angel et al., 2002a,b). The wider the ratio of Ca to $InsP_6$ the higher is the risk of formation of insoluble complexes (Selle and Ravindran, 2007).

Phytate may also form complexes with proteins, amino acids and starch in the digestive tract. Binary protein-phytate complexes are formed below protein isoelectric points by electrostatic attractions between negatively charged $InsP_6$ or phytate and proteins carrying a net positive charge (Selle et al., 2012). The isoelectric point of proteins in cereal grains may be sufficiently high to permit these complexes to persist in the small intestine (Selle et al., 2012). Above their isoelectric point, proteins carrying a net negative charge are connected with $InsP_6$

or phytate by divalent cationic bridges to form ternary protein-phytate complexes (Selle et al., 2012). This may occur in the small intestine of broilers. Starch may interact directly with phytate or via binding proteins that are closely associated with starch granules (Selle et al., 2012). In addition, phytate may inhibit the efficacy of digestive enzymes such as α-amylase, trypsin and pepsin by interactions with the enzyme or the substrate (Selle et al., 2000). Hence, phytate may also impair energy and protein utilization in broilers. For further details on these aspects the reader is referred to the reviews of Selle et al. (2000, 2012).

Phytate has two faces: it is a mild antinutrient and at the same time a valuable nutrient. In order to release its nutritive effect in the gut of poultry, phytate has to be degraded rendering P and other bound nutrients available. Phytate degradation depends on accessibility and solubility, which may vary between feed ingredients and under different assay conditions in vitro. As those can only incompletely simulate the varying physiological conditions prevailing in the digestive tract the degradation of feed phytate within different segments of the broilers' digestive tract needs to be investigated.

1.4 PHYTASE

To address the environmental relevance of poultry production several strategies have been developed to reduce P excretion. As a first step, phase feeding was implemented to adapt the non-phytate bound dietary P content accurately to birds' age-dependent P requirement (O'Rourke et al., 1952, Waldroup et al., 1963; Sauveur, 1978). However, phase feeding may not minimize phytate output and InsPs may be important for the germination process and performance of the plant. Different pretreatment processes have been used to eliminate phytate from pig feed, such as soaking, germination, mechanical removal, extraction, cooking, fermentation or heat treatment, which was already reviewed elsewhere (Sandberg and Andlid, 2002). However, most of these approaches depend on intrinsic feed phytase activity and thus on plant species, cannot ensure a satisfactory level of efficacy, are related with a removal or destruction of other nutrients or are simply impracticable in poultry feeding. A strategy which does not imply these aspects is to promote the enzymatic degradation of phytate by phytases throughout the passage of the digestive tract. The most effective, practical and common tool for this purpose in the poultry industry today is the supplementation of microbially derived phytases to the feed.

Definition, classification and nomenclature

Phytases ($InsP_6$ phosphohydrolases) are by definition enzymes which are able to catalyze the hydrolytic cleavage of $InsP_6$ to $InsP_5$ and inorganic phosphate (P_i) (Sandberg and Andlid, 2002). As phytases are not specific for $InsP_6$, phytases can further catalyze the stepwise hydrolysis of $InsP_5$ via $InsP_4$, $InsP_3$ and $InsP_2$ to $InsP_1$ or, in some cases, down to *myo*-inositol. Lower InsPs may also be dephosphorylated by other phosphatases, which do not hydrolyse $InsP_6$. The generic term "phytase" refers to a diverse group of enzymes which differ in catalytic properties and mechanisms, requirements, structures, sizes and origins (Mullaney and Ullah, 2003). Hence, nature evolved different strategies to cleave phosphate groups from $InsP_6$ by diverse organisms and under different conditions (Mullaney and Ullah, 2007). Four classes of phosphatases able to hydrolyse $InsP_6$ have been reported to date, grouped on the basis of catalytic mechanisms: Histidine acid phosphatase, β-propeller phytase, cysteine phosphatase and purple acid phosphatase (Mullaney and Ullah, 2007). Within these subgroups enzymes show common characteristics but there is further diversity and characteristics of one phytase cannot be assumed for another. In poultry nutrition, the majority of supplemented phytases refer to histidine acid phosphatases of bacterial or fungal origin, since these are supposed to be highly effective in the anterior segments of the digestive tract. At present, ten phytase products are authorized for the use as feed additives for broilers in the EU (Table 1).

TABLE 1. Microbial phytases authorised for the use as broiler feed additives in the EU according to the European Food Safety Authority (EFSA)[1]

Identification number	Name of the holder of authorisation	Product (Trade name)	Production organism	Minimum content (U/kg feed)[2]
4a1	Adisseo	3-phytase EC 3.1.3.8 (Rovabio™ Phy)	*Penicillium funiculosum* (CBS 111433)	350
4a5	AB Enzymes	6-phytase EC 3.1.3.26 (Quantum®)	*Pichia pastoris* (DSM 15927)	500
4a6	DSM Nutritional Products	6-phytase EC 3.1.3.26 (Ronozyme® NP)	*Aspergillus oryzae* (DSM 17594)	1,500
4a12	Roal Oy	6-phytase EC 3.1.3.26 (Finase® EC)	*Trichoderma reesei* (CBS 122001)	250
4a16	Huvepharma AD	6-phytase EC 3.1.3.26 (Optiphos®)	*Pichia pastoris* (DSM 23036)	125
4a18	DSM Nutritional Products	6-phytase EC 3.1.3.26 (Ronozyme® Hiphos)	*Aspergillus oryzae* (DSM 22594)	500
4a19	Roal Oy	6-phytase EC 3.1.3.26 (Quantum® Blue)	*Trichoderma reesei* (CBS 126897)	250
4a1600	BASF SE	3-phytase EC 3.1.3.8 (Natuphos®)	*Aspergillus niger* (CBS 101.672)	275
4a1640	Danisco Animal Nutrition	6-phytase EC 3.1.3.26 (Phyzyme® XP)	*Schizosaccharomyces pombe* (ATCC 5233)	250
4a1641(i)	DSM Nutritional Products	6-phytase EC 3.1.3.26 (Ronozyme® P)	*Aspergillus oryzae* (DSM 14223)	250

[1]Adapted and modified from the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003, which is published on the European Commission's website. Additional information was from publications on the EFSA's website (EFSA, 2005, 2007, 2008a,b, 2009a,b, 2010, 2011, 2012, 2013)

[2]Activities of different products are in part determined under different conditions

Based on stereospecificity of $InsP_6$ hydrolysis, three types of phytases have been recognized by IUPAC-IUB: 3-phytases (1-phytases based on L-numbering) (E.C.3.1.3.8), 4-phytases (6-phytases based on L-numbering) (E.C.3.1.3.26) and 5-phytases (E.C.3.1.3.72) (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1973). The occurrence of 1-phytases based on D-numbering or 2-phytases has not been described to date, but cannot be ruled out considering the high diversity of phytases. InsPs which are dephosphorylated at the C-2 position of the *myo*-inositol ring are present within animal cells (Irvine and Schell, 2001), which suggests existence of a 2-phytase (Greiner, 2010). This classification refers to the major position of the initial dephosphorylation of $InsP_6$ *in vitro*. 3-phytases initially dephosphorylate $InsP_6$ on position D-3 (L-1), whereas 4-/6- and 5-phytases prefer the position D-4 (L-6) and D-5 (L-5) respectively (for nomenclature see Figure 1) (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1973). 5-phytase activity was reported for *S. ruminantium*, *B. pseudocatenulatum* and the alkaline phosphatase of *Lily pollen* (Barrientos et al., 1994; Puhl et al., 2008; Haros et al., 2009). As reviewed by Konietzny and Greiner (2002) phytases from microorganisms are considered to be 3-phytases, whereas seeds of higher plants are considered to contain 4-/6-phytases. As the major $InsP_5$ generated by phytases from *S. cerevisiae, Pseudomonas*, *K. terrigena* and *A. niger* has been identified as D-Ins(1,2,4,5,6)P_5 these phytases are 3-phytases and fit into this general consideration (Konietzny and Greiner, 2002). The major $InsP_5$ generated by phytases from rye, barley, *Triticum spelta*, oat, wheat bran, rice and mung bean was identified as D-Ins(1,2,3,5,6)P_5; Thus, they represent 4-/6-phytases and also fit into the general consideration (Konietzny and Greiner, 2002). However, this rule does not apply generally, as 3-phytase activity was also found for soybean (Phillippy and Bland, 1988) and the major $InsP_5$ formed by phytases from *E. coli*, *P. lycii* and *B. pseudocatenulatum* was identified as D-Ins(1,2,3,4,5)P_5 (Van der Kaay and Van Haastert, 1995; Greiner et al., 2000a; Haros et al., 2009). Moreover, especially in plant seeds multiple forms of phytases have been found which may show different stereospecific degradation pathways of $InsP_6$, as reported for phytases from lupin or wheat (Lim and Tate, 1973; Konietzny and Greiner, 2002). Thus, major and minor degradation pathways of $InsP_6$ were reported for various types of phytases (Figure 2-4). The order of activity of different isoenzymes may vary depending on the used *in vitro* conditions, and on the different conditions in the digestive tract. Hence, a generalization relative to the initial attack to the susceptible phosphor-ester bounds is hasty (Greiner et al., 2000a) and has to be reconsidered.

Clarification still is needed regarding nomenclature of 4-/6-phytases. Confusion occurs in literature as bacterial phytases, such as *E. coli* phytase, which preferably generate D-Ins(1,2,3,4,5)P_5 (Greiner et al., 2000a), are classified as 6-phytases. But according to IUPAC's protocol, phytases that generate D-Ins(1,2,3,5,6)P_5, such as the majority of plant phytases, are 4-/6-phytases. IUPAC-IUB suggested to use the numbering of the D-configuration for *myo*-inositol (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1973). Therefore, to avoid misconceptions the aforementioned plant 4-/6-phytases should be termed 4-phytases and the term 6-phytases should be avoided for this type of phytase. Enzymes which predominantly generate D-Ins(1,2,3,4,5)P_5, such as the investigated phytase from *E. coli*, should instead be called 6-phytases.

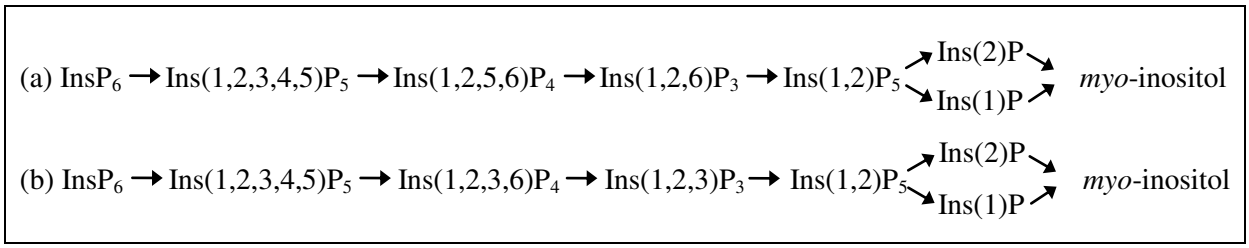

FIGURE 2. Major (a) and minor (b) pathway of $InsP_6$ degradation by phytases from wheat bran of *T. aestivum*, without separation of enantiomers (Nakano et al., 2000)[1]

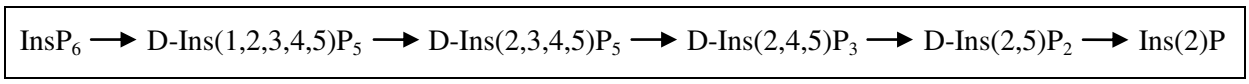

FIGURE 3. Major pathway of $InsP_6$ degradation by a phytase from *E. coli* (Greiner et al., 2000a)[2]

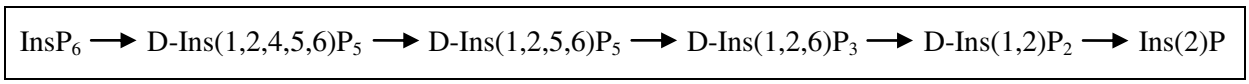

FIGURE 4. Major pathway of $InsP_6$ degradation by a phytase from *A. niger* (Greiner et al., 2009)

[1] Lim and Tate (1973) showed that a wheat phytase predominantly produces the D-Ins(1,2,3,5,6)P_5, D-(1,2,3,6)P_4 and D-(1,2,3)P_3 isomers. Tomlinson and Ballou (1962) suspected the L-Ins(1,2,3,5,6)P_5 and L-(1,2,5,6)P_4 isomers to be formed by wheat phytase. Later on, Ins(1,2,4,5,6)P_5 was identified as a secondary $InsP_5$ isomer formed by purified Sigma wheat phytase (Bohn et al., 2007).

[2] Ins(1,2,4,5,6)P_5 and Ins(1,2,3,4)P_4 were shown as minor degradation products of the investigated *E. coli* phytase (Greiner et al., 1993).

Intrinsic plant phytases

Phytases are known to be widely present in plants, animals and microorganisms. In plants, phytase has been detected in roots, pollen and especially in seeds. A high phytase activity was reported for rye (4132-6127 U/kg), triticale (1475-2039 U/kg), wheat (915-1581 U/kg), barley (408-882 U/kg) and wheat by-products (up to 5345 U/kg), whereas no or only little phytase activity was detected in corn (0-46 U/kg), oats (0-108 U/kg), sorghum (0-76 U/kg) and oilseeds (e.g. extracted soybean 0-120 U/kg) (Eeckhout and De Paepe, 1994). The phytase in dormant, dry seeds is inactive, but activity rapidly increases during imbibition and germination. In ungerminated grains, intrinsic phytase is predominantly localized in the aleurone layer (Peers, 1953; Fretzdorff and Weipert, 1986), which explains the often reported high phytase activity in several wheat milling by-products such as wheat bran or wheat middlings. High variation in phytase activity within plant species is related to differences in genetics, age, harvest year, growing, drying, processing and storage conditions (Barrier-Guillot et al., 1996; Cossa et al., 2000; Steiner et al., 2007; Singh, 2008, Blaabjerg et al. 2010a).

Details on molecular and catalytic properties of different plant phytases were reviewed elsewhere (Konietzny and Greiner, 2002). In brief, the pH optimum of most plant phytases ranges between 4.3 and 7.5 with dramatically decreasing stability at pH values below 4 and above 7.5 (Konietzny and Greiner, 2002). As plant phytase is susceptible to proteolytic digestion, it may be inactivated during the passage through the acid proventriculus and gizzard. Thus, activity within the broilers' digestive tract probably is limited to the short retention in the crop. Practical relevance of plant phytase in pelletized diets may be restricted by heat lability. In purified form, plant phytases are partially or completely irreversible denaturated at temperatures occurring during pelleting or processing (Konietzny and Greiner, 2002). Although heat stability for intrinsic phytase in wholemeal wheat and seeds was reported to be higher than for extracted wheat phytase (Peers, 1953; Ma and Shan, 2002), different studies reported an inactivation of wheat phytase during pelletizing with application of steam (Jonglbloed and Kemme, 1990; Larsen et al., 1999; Blaabjerg et al., 2010a; Carlson and Poulsen, 2003). However, the use of plant phytase is not generally limited in practice since many broiler producers use unpelletized diets or whole grains.

Endogenous and microbiota-associated phytases

Besides dietary phytases, endogenous and microbiota-associated phytases may be active in broilers. Endogenous phytase was reported intracellularly, in the intestinal brush border

membrane and in the erythrocytes of chicken (Martin and Luque, 1985; Maenz and Classen, 1998; Cho et al., 2006). It is known that broilers are able to hydrolyse a varying proportion of phytate in their digestive tract even when diets without detectable phytase activity are fed. The detected endogenous mucosal phytase may contribute to this hydrolysis in addition to microbiota-associated phytases. However, endogenous phytase (and other phosphatase) activity associated with the mucosa is often dismissed as having little importance in $InsP_6$ degradation in the digestive tract of poultry (Selle and Ravindran, 2007). In regard to microorganisms, especially rumen-, soil- and mycorrhizal microorganisms have been studied regarding their phytase activity. Studies which investigated phytase activity from microorganisms of poultry's digestive tract are rare. However, hindgut microbiota has been suggested as a potentially important player in phytate hydrolysis in poultry (Kerr et al., 2000). The efficacy of endogenous mucosal and microbiota-associated phytases in the digestive tract of broilers will be discussed in more detail in Chapter 3.

Commercial microbial phytases

In terms of microorganisms, phytase is known to be naturally secreted by a range of fungi, bacteria and yeasts. Most microorganisms only produce intracellular phytases (Konietzny and Greiner, 2002). Extracellular phytase is predominantly produced by species of *Filamentous fungi* (Shieh and Ware, 1968; Gargova et al., 1997). To date, the only bacteria showing extracellular phytase activity are those of the genera *Bacillus* and *Enterobacter*; phytases derived from *E. coli* have been reported to be periplasmatic enzymes (Konietzny and Greiner, 2002). Hence, the majority of commercially available feed phytases are derived from fungi or, more recently, bacteria, such as *A. niger* or *E. coli* (Selle et al., 2010). Classically, microbial enzymes were produced by wild type or classical modified hosts (Paloheimo et al., 2010). Wild type organisms usually produce too low levels of phytase which are not suitable for industrial application (Greiner, 2010). Classical modified strains are derived from natural isolates with desired characteristics and have been subjected to several rounds of mutagenesis and screening for high enzyme productivity over decades (Paloheimo et al., 2010). At present, the fermentative production processes mainly depend on genetically modified organisms (Selle et al., 2010). With gene technology genes expressing phytases with desired characteristics for the application are inserted into production organisms. Maximal expression level of the gene is achieved by insertion of multiple gene copies and/or by placing the desired gene under the control of a strong promoter (Paloheimo et al., 2010). After fermentation, purification steps ensure that no genetically modified recombinant DNA is

detected in the final product (Adeola and Cowieson, 2011). Ronozyme® P e.g. is a preparation of a 6-phytase produced by *Aspergillus oryzae* (DSM 14223) carrying a gene coding for phytase from *Peniophora lycii* (EFSA, 2010).

Properties of microbial phytase primarily determine its efficacy throughout the passage of the digestive tract and thus suitability as feed supplement. Generally, the majority of microbial phytases have a higher temperature optimum (50-77°C) than most plant phytases (35-58°C) and are more heat stable (Konietzny and Greiner, 2002). Specific types, such as an *A. fumigatus* derived phytase, are able to refold properly after denaturation by heat treatment (Wyss et al., 1998). Furthermore, *in vitro*, most microbial phytases are stable at pH above 8.0 or below 3.0 (pH optimum ranges between 2.2 and 8.0) and show higher stability in the presence of pepsin and pancreatin compared to their plant counterpart (Konietzny and Greiner, 2002). Thus, microbial phytases not only withstand better inactivation during the pelleting process, but also may retain higher activity in the proventriculus and gizzard and following segments. The crop, proventriculus and gizzard were reported as the favourable site of action of microbial phytases, due to their pH environment (Yu et al., 2004). Low activity was found in the small intestine, probably due to less favourable pH and proteolysis (Yu et al., 2004). However, properties of microbial phytases of different origin vary. Phytase from *A. niger* has two pH optima (2.2; 5.0-5.5), whereas phytase from *E. coli* or *B. subtilis* has a single pH optimum at 4.5 and 6.0-7.5, respectively (Konietzny and Greiner, 2002). The *Aspergillus* enzyme is more resistant to trypsin but less resistant to pepsin and pancreatin than phytase from *E. coli* (Konietzny and Greiner, 2002). When diets containing microbial phytases were pelletized at 70°C, an *E. coli* derived phytase retained higher activity than different *Aspergillus* phytases (Simon and Igbasan, 2002). *E. coli* derived phytase had a higher K_m, but a higher k_{cat} value and kinetic efficiency (k_{cat}/K_m) compared to *Aspergillus* derived phytase (Konietzny and Greiner, 2002).

Differences in properties between microbial phytases may result in differences in efficacy under the varying conditions of the digestive tract. In this context, residual phytase activity from *E. coli* was 97%, 93%, 97%, 87% and 80% after incubation in digesta supernatants from the crop, stomach, duodenum, jejunum and ileum (Simon and Igbasan, 2002). Residual phytase activity from *Aspergillus* was in a range between 93-99%, 57-68%, 90-96%, 43-90% and 55-81%, respectively, depending on the production organisms and originating strain of the phytase gene (Simon and Igbasan, 2002). Activities and efficacy may further differ when enzymes pass the different segments consecutively.

Determinants of phytase efficacy

Apart from the conditions in the digestive tract such as pH, temperature, activity of proteolytic enzymes and source and characteristics of the specific phytases various other factors have been identified to influence efficacy and activity of phytases: genetics, age of birds, substrate variability or other dietary factors (Selle and Ravindran, 2007; Singh, 2008). Different factors will be discussed later in more detail. In the following, the dietary factors that received the major attention are briefly mentioned.

The most consistent, and probably the strongest, dietary factor is the aforementioned Ca concentration and Ca:$InsP_6$ ratio (Lei and Porres, 2007). This primarily influences phytate solubility. Likewise, the level of dietary P_i may reduce phytate degradation by phosphatases due to end-product inhibition (Shieh et al., 1969). These two dietary factors will be discussed in more detail in Chapter 3. Use of organic acids, such as citric, formic or lactic acid, may increase phytate degradation in the digestive tract. The mechanism behind is still unclear (Liem et al., 2008). In part, the effects of organic acids on phytate P utilization might result from a change of pH in the gastrointestinal tract to a value more favourable for phytase (Liem et al., 2008). Boling et al. (2000) suggested that citric acid chelated Ca which reduced the formation of insoluble Ca-phytate complexes, and thus, susceptibility of phytate for phytases. Further, dietary vitamin D or its metabolites may improve phytate degradation in the digestive tract. This was attributed to increased synthesis or activity of endogenous mucosal phytase (Davies et al., 1970). Vitamin D also stimulates Ca and P absorption. Enhanced Ca absorption may diminish the formation of insoluble Ca-phytates, and thus, indirectly increase phytate degradation (Ravindran et al., 1995). In addition, Mitchell and Edwards (1996) suggested that vitamin D increases the uptake of the liberated P_i once phytate is degraded by phytase.

The combination of supplemented carbohydrases or proteases with phytase may show an additive effect on $InsP_6$ hydrolysis (Lei and Porres, 2010; Woyengo and Nyachoti, 2011). The main function of supplemented carbohydrases is to hydrolyse complex non-starch polysaccharides (NSP) of plant feedstuffs (Castillo and Gatlin, 2015). In plant seeds, phytate is located intracellularly (Prattley and Stanley, 1982). Insoluble NSPs are present in the cell wall (Simon, 2000). Consequently, when these insoluble NSPs are hydrolysed, phytate and other minerals may be exposed to phytase and other digestive enzymes and liberated for absorption. Furthermore, soluble NSPs are known to produce high viscosity and it is assumed that elevated digesta viscosity cause impaired nutrient availability (Simon, 2000). Therefore, their partial hydrolysis may reduce digesta viscosity and increase absorption of nutrients

liberated by phytase. More than 80% of the global carbohydrase market is accounted for by xylanase and glucanase (Adeola and Cowieson, 2011). The substrates, arabinoxylans and β-glucans are particularly located in the cell walls of the starchy endosperm and aleurone layer of cereal grains (Paloheimo et al., 2010). Phytate was previously described to be in part present in these fractions. Supplemented xylanase may be most efficient in diets based on wheat, rye, triticale or maize, because the major NSPs in these feedstuffs are arabinoxylans (Woyengo and Nyachoti, 2011), whereas β-glucans dominate in barley and oats (Paloheimo et al., 2010). The most effective combination is achieved when different carbohydrase product are used together because e.g. some xylanases target soluble and other insoluble arabinoxylans (Castillo and Gatlin, 2015). In addition, supplementation of phytase plus a preparation containing carbohydrases that target all the major NSPs in the diet (in cereal grains and oilseed meals), may result in improved nutrient utilization than supplementation of phytase plus carbohydrases that only target the major NSP in the dominating cereal (Woyengo and Nyachoti, 2011). However, the response to supplementation of a combination of these enzymes is variable and depends on several factors (Woyengo and Nyachoti, 2011). Overall, the influence of different factors on InsP degradation in the digestive tract of broilers, the mechanisms behind and their interactions are not well understood and quantified.

Beyond hydrolysis of $InsP_6$, the supply of available P depends on further degradation of lower InsPs. Phytases of different origin vary in $InsP_6$ degradation pathways and other in vitro properties. Those properties may vary under the conditions along the digestive tract. As in vivo properties affect the enzyme efficacy, $InsP_6$ hydrolysis and appearance of lower InsPs in the presence of different types of dietary phytases should be investigated in different segments of the digestive tract. Better knowledge about dietary key factors, affecting InsP degradation along the digestive tract, and their interactions, is needed. These information may provide a helpful tool to maximize degradation of feed phytate and efficacy of dietary phytases in the digestive tract of broilers.

CHAPTER 2

OVERVIEW AND RESEARCH QUESTIONS OF THE INCLUDED MANUSCRIPTS

2 OVERVIEW AND RESEARCH QUESTIONS OF THE INCLUDED MANUSCRIPTS

Against the background outlined in Chapter 1 the overall aim of this doctoral project was to contribute to filling the knowledge gap in respect to InsP degradation in the digestive tract of broilers. In line with this, a compilation of five manuscripts is included investigating $InsP_6$ hydrolysis and appearance of lower InsPs in different segments of the digestive tract of broilers as influenced by different dietary factors. Chapter 4 contains the five manuscripts which objectives can be briefly characterized as follows.

MANUSCRIPT 1: Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplemented phytases in different segments of the digestive tract of broilers

It is still unknown which positional InsPs are formed by different phytases and other phosphatases in the digestive tract of broilers. Therefore, the objective of this first study was to characterize and understand better $InsP_6$ hydrolysis and formation of lower InsPs in different segments of the digestive tract. Phytases of different origin show different properties and InsP degradation pattern *in vitro*. Under the complex and variable physiological conditions those properties and the appearing InsP pattern may be different. Thus, the second objective was to investigate the $InsP_6$ degradation pattern of different phytase additives and their effectiveness in releasing phosphate in different segments of the digestive tract of broilers and to compare the findings with known *in vitro* properties.

The manuscript was published in the *Journal of Nutritional Science.*

MANUSCRIPT 2: Effects of the composition of the basal diet on the evaluation of mineral phosphorus sources and interactions with phytate hydrolysis in broilers

Determined P availability from a mineral P source may depend on the type of basal diet used. Results obtained by using semi-synthetic diets may be different when compared with those from phytate-containing diets. Different results may also be found by using maize- or wheat-based diets which may show different $InsP_6$ hydrolysis due to different intrinsic phytase activity. Mineral P, usually present in broiler diets, may interact with phytases and other phosphatases present in the digestive tract by product inhibition. Therefore, supplemented mineral P could influence InsP hydrolysis from the basal diet in P supplementation studies. One major objective of this study was to determine the availability of P from mineral

phosphates by using semi-synthetic and practical-type basal diets. The second objective was to investigate whether supplemental mineral P influences InsP hydrolysis with consequences for calculation of P availability of a mineral supplement.

The manuscript was published in *Poultry Science*.

MANUSCRIPT 3: Interactions between supplemented mineral phosphorus and phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers

The previous studies (Manuscript 1 and 2) described the separate influence of phytase or mineral P supplements on $InsP_6$ hydrolysis and the InsP pattern in the gut. But, mineral P in the diet may also affect the efficacy of supplemented phytase. High doses of phytase beyond the current industry standard levels may let a lower part of dietary InsP appear undegraded in the ileum of broilers than standard levels of phytase. The decrease in phytase efficacy by mineral P was supposed to be lower for such high compared to the standard levels of phytase. Therefore, the aim of the third study was to gain a better understanding of these interactions. The objectives were to investigate the effects of supplemented phytase (a standard and a very high level), a mineral P supplement, and their interactions on $InsP_6$ hydrolysis and the appearance of lower InsPs in the duodenum/jejunum and lower ileum of broilers.

The manuscript was published in *Poultry Science*.

MANUSCRIPT 4: Effect of enzyme supplementation in diets containing microwave treated or untreated wheat on inositol phosphates in the small intestine of broilers

Results on the relevance of intrinsic plant phytase for P digestibility in poultry are inconsistent. This may in part be related to the use of different grain species or varieties to achieve differences in intrinsic phytase activities. This confounds intrinsic phytase activity with other factors such nutrient contents or structures which may also affect InsP degradation. Therefore, in this study, differences in intrinsic phytase activities were achieved by microwave treatment of wheat. Microwave treatment may also cause structural changes, affecting $InsP_6$ accessibility and thereby the response to supplemented phytase and xylanase in InsP degradation. Therefore, the objectives of this study were to evaluate the separate and interactive effects of microwave treatment in wheat and phytase supplemented alone or together with xylanase on $InsP_6$ hydrolysis and InsPs in the small intestine of broilers.

The manuscript was published in *Animal Feed Science and Technology*.

MANUSCRIPT 5 (draft): Effect of enzyme or mineral P supplementation in diets differing in intrinsic phytase activity on inositol phosphates in the crop of broilers

Manuscript 5 describes the influence of different dietary factors on InsP degradation in the crop of broilers. In theory, the crop is the major site of action of most dietary phytases. Better knowledge about InsP degradation by dietary phytases and different influencing factors in this segment may deliver information to increase their efficacy in broilers. It is still unknown whether microbial and intrinsic plant phytase show synergistic or additive effects on InsP degradation in the crop. Xylanase may further increase the efficacy of supplemented phytase in wheat-based diets. In contrast, mineral P supplements may reduce the efficacy of microbial phytase in the crop. In experiment 1 of this manuscript, the effects of supplemented phytase
(a standard and a very high level), a mineral P supplement, and their interactions on $InsP_6$ hydrolysis and the appearance of InsPs in the crop were studied. Experiment 2 was conducted to study the separate and interactive effects of microwave treatment in wheat and phytase supplemented alone or together with xylanase on $InsP_6$ hydrolysis and lower InsPs in the crop.

This is a draft version of a manuscript still to be submitted.

Results shown in Manuscript 3 and Manuscript 5 (experiment 2) and in Manuscript 4 and Manuscript 5 (experiment 1) are based on the same animal study, respectively.

CHAPTER 3

GENERAL DISCUSSION

3 GENERAL DISCUSSION

In the digestive tract of avian species, dietary, endogenous mucosal or microbiota-associated phytases and other phosphatases can contribute to InsP degradation. The high efficacy of supplemental microbial phytases in broilers has been frequently reported in literature. However, apart from the environmental perspective, high efficacy of intrinsic plant, endogenous mucosal and microbiota-associated phytases and phosphatases is of economical interest. High InsP hydrolysis by those enzymes may reduce the need to supplement expensive mineral P and microbially derived enzymes. But literature on the activity and efficacy of those different types of phytases in consecutive segments of the digestive tract of broilers is rather scarce. Based on the findings from the studies of the present thesis the contribution and efficacy of endogenous mucosal and microbiota-associated enzymes to phytate hydrolysis in different segments of the digestive tract of broilers is debated in this general discussion. Factors which influence phytate hydrolysis will also be discussed. Since the included manuscripts described the effect of dietary factors on lower InsPs, the relevance of lower InsPs in the digestive tract is also considered in this general discussion. The last part will deal with the consequences of the reported findings for P evaluation in poultry.

3.1 METHODICAL CONSIDERATIONS

Some methodological aspects can be of relevance and constraints for the results of the present work and will be discussed herein.

Ad libitum-fed birds may adapt a habit of letting feed bypass the crop (Svihus, 2014). They eat small amounts approximately every half an hour and discourage the use of the crop as a storage organ (Svihus et al., 2013; Svihus, 2014). It is not known which proportion of total digesta from oesophagus entered and bypassed the crop in the present work. Therefore, it cannot be ruled out that the measured $InsP_6$ hydrolysis in the crop overestimated the contribution of the crop to $InsP_6$ hydrolysis measured in the more posterior segments. The only situation the crop holds substantial amount are after periods of fastening, when birds are hungry and eat more (Savory, 1985) because the storage capacity of the gizzard is usually limited to a maximum of 5 to 10 g of feed (Svihus, 2014). In quails fasted for two hours, then allowed access to feed for 20 min and then killed in intervals during the subsequent two hours, maximum amount of undiluted and diluted food in crop and gizzard are generally greater than in ad libitum-fed quails (Savory, 1985). Correspondingly, Svihus (2014) reported a transient storage of large quantities of feed in the crop, when birds are trained to intermittent

feeding. In the present work, birds had access to feed for one hour after a fasten period of one hour. Perhaps, they consumed large amounts of feed after the fasten period and stored substantial amounts in the crop. A retention time in the proventriculus/gizzard between 20 min and 60 min (Dänicke et al., 1999) indicates that the majority of digesta in the small intestine originated from the feed intake before fastening in the present work. This suggests that the measured $InsP_6$ hydrolysis in the crop may not comply with the $InsP_6$ hydrolysis which really occurred in the crop before the digesta passed the more posterior segments. However, it can demonstrate the potential for $InsP_6$ hydrolysis in the crop. The crop fill and retention time effects may also explain that $InsP_6$ hydrolysis calculated in the crop (Manuscript 5) was higher compared to the duodenum/jejunum (Manuscript 3) of birds fed diets with monocalcium phosphate (MCP) and 500 FTU/kg phytase. Moreover, the retention time of digesta in the crop in the present work did not exceed one hour when birds were killed. It cannot be ruled out that $InsP_6$ hydrolysis in the crop is higher with longer retention time. In regard to the samples from the crop and proventriculus/gizzard it is also possible that further hydrolysis occurred between sampling of digesta of body temperature and cooling down to temperatures at which phytases are inactive in the freezer. In the present work, crop and gizzard samples were put into the freezer as fast as possible subsequent to sampling. Shock-freezing of the samples in liquid nitrogen could prevent further hydrolysis after sampling.

$InsP_6$ hydrolysis was not calculated for the proventriculus/gizzard because this segment clearly contained particles of different sizes. Particles of different sizes are presumed to have variable retention time in the gizzard (Svihus et al., 2002). This indicates that they were not accurately represented by the marker. Moreover, soluble and insoluble fractions may differ in retention time as shown by a faster passage rate of a soluble compared to an insoluble marker through the gizzard (Vergara et al., 1989). Svihus et al. (2002) further suggested that high-density particles could fall to the bottom, because opening of the gizzard is on the dorsal side. This could impair the passage rate of high dense particles. The presence of the marker and $InsP_6$ in different fractions linked with different flow rates precludes an interpretation in regard to changes in $InsP_6$ hydrolysis between the crop and proventriculus/gizzard or the proventriculus/gizzard and posterior segments. Results of Sooncharenying and Edwards (1993) already indicated a dilution of phytate and disappearance of the marker chromic oxide in the crop and gizzard of chicken. Kerr et al. (2000) also supposed differential passage rates for $InsP_6$ and acid insoluble ash in the gizzard. In contrast, particles that enter the duodenum are relatively homogenous, the majority being smaller than 0.1 mm and 90% being smaller

than 0.3 mm (Hetland et al., 2002). This homogeneity points to a more homogeneous flow of the small feed particles and the marker TiO_2, which has a relatively low specific gravidity, in the small intestine. Selective retention is unlikely to occur in the crop due to a lower peristalsis and moisture content. However, in Manuscript 5 the calculated negative $InsP_6$ hydrolysis in the crop of birds fed the basal diet without MCP indicates a slightly deviating flow of marker and $InsP_6$.

In addition, it has to be taken into consideration that concentrations of InsPs, P and Ti might be influenced by retrograde movement of digesta and microorganisms, as has been described for all segments of the digestive tract in broilers (Sacranie et al., 2012b).

It became apparent that birds consumed litter material in experiments described in Manuscript 1, 2, 4 and 5 (experiment 1). This probably includes the intake of associated excreta containing higher concentrations of Ti and different concentrations of $InsP_6$ and P compared to the diet. It cannot be ruled out that calculated $InsP_6$ hydrolysis overestimated $InsP_6$ hydrolysis occurring in the digestive tract. Calculations from analysed acid detergent fibre concentrations of feed, crop and gizzard contents, litter material (wood shavings and excreta) and wood shavings (Annex 1) indicated an average litter concentration of 6% and 75% and excreta concentration 3% and 32% in the crop and gizzard, respectively. In the gizzard, wood shavings seem to be retained and accumulate. Whether bound excreta is retained to the same extent, is uncertain, but it can be speculated that high amounts of litter material in the gizzard also influence calculated $InsP_6$ hydrolysis in this segment. Whether the low intake estimated in the crop remarkably influenced calculated $InsP_6$ hydrolysis in the crop and intestinal segments, where particle and marker flow seems to be more similar, needs to be clarified. However, in the present work, the birds may have consumed high amounts of litter material in the fastening period. It cannot be ruled out that wood shavings were retained in the gizzard and faster flowing excreta influenced the Ti and nutrient concentrations in the small intestine at sampling time.

The detected concentration of any InsP isomer represents a net value. It contains the amount originating from feed and not degraded until the investigated segment, the amount originating from activity of phosphatases and perhaps the amount which was absorbed or secreted or both. Therefore, a calculation of hydrolysis compared to the feed based on the marker as done for $InsP_6$ is not possible for specific lower InsPs.

During the passage of the digestive tract intense secretion and absorption processes occur. Calculated dry matter (DM) digestibility reflected intense secretion processes in the

proventriculus/gizzard and duodenum due to stomach, bile, and pancreatic secretions and continuous nutrient absorption in the jejunum and ileum (Dänicke et al., 1999). These processes are also reflected by de- and increased InsP concentrations per g of DM between the segments. This stands against an interpretation of changes of InsP concentrations between the segments. However, calculations of InsP concentrations in relation to Ti concentrations (data not shown) proved that differences in InsP concentrations between treatment groups were not affected by DM digestion. This allows an interpretation in regard to differences in InsP concentrations between the treatment groups.

3.2 $InsP_6$ hydrolysis in different segments of the digestive tract of broilers

Digestion includes mechanical and chemical breakdown of ingested feed constituents. Large feed particles are physically and enzymatically broken down to smaller ones and molecules which can be absorbed. Enzymes of endogenous, feed and microbial origin are involved in these processes. In regard to phytate very different conditions in different compartments of the digestive tract of broilers determine the digestion process. To better understand these processes the following subchapters will discuss the extent of $InsP_6$ hydrolysis and enzymes involved in different segments of the digestive tract.

3.2.1 Crop

The crop, a unique organ of avian species, primary acts as a storage organ. It is assumed that the crop does not have any direct nutritional role, as it does not secrete enzymes and considerable absorption does not occur (Svihus, 2014). However, some degradation of feed constituents by salivary or microbial enzymes in this segment was reported (Denbow, 2000). The microbiota in the crop may also play a role in phytate degradation in broilers as indicated by one study of Kerr et al. (2000). These authors found $InsP_6$ hydrolysis in the crop of broilers fed a maize-soybean meal-based diet irrespective of phytase supplemented or not. However, they did not report the level of $InsP_6$ hydrolysis. In the present work a negligible $InsP_6$ hydrolysis was measured in the crop of broilers fed maize-based diets without detectable phytase activity (up to 9%; Manuscript 1 and 5). This is in accordance with the marginal $InsP_6$ hydrolysis of a maize-soybean meal-diet without supplemental phytase found during incubation in water at 38°C (Ton Nu et al., 2014). These results indicate that phytate hydrolysis by feed- and microbiota-associated phytases in the crop plays a minor role in the overall phytate hydrolysis in broilers when the diet contains no detectable phytase activity. As

discussed later in more detail solubility and accessibility of phytate in the maize and soybean meal did not limit hydrolysis of the major part of feed $InsP_6$ in the crop in the present studies (Manuscript 5). Thus, it can be concluded, that the activity of feed- and microbiota-associated phytases or the abundance of phytase-producing microorganisms, or both, is low in the crop of birds fed maize-soybean meal-based diets. This is supported by the relatively low phytase activity detected in the crop digesta of 20-day old laying hens fed a maize-soybean meal based-diet (Marounek et al., 2008). These authors incubated phytate with digesta from the crop and small intestine. Per g of digesta, an $InsP_6$ disappearance of 7 and 14 µmol $InsP_6$/h was found in the crop and small intestine, respectively. Expressed per g DM of digesta this means a much lower phytase activity in the crop compared to the small intestine.

The crop has important functions in regard to phytate degradation in the more posterior digestive tract. Mucus which is secreted by the epithelia of the crop moistures the digesta (Denbow, 2000). Hydration and warming up may be favourable for bacterial or dietary enzyme activities (Svihus et al., 2002). Enzymatic breakdown of plant tissues surrounding phytate may increase the accessibility of phytate for phytases further down the digestive tract. These "preparation" processes of feed for mechanical and enzymatic digestion are obviously more decisive than direct $InsP_6$ hydrolysis in the crop when diets without detectable phytase activity are fed. Cropectomy could give information about the role of the crop in phytate degradation in broilers. An early study in laying hens reported no significant effect of the removal of the crop on bone breaking strength or tibia ash (Bayer et al., 1975; Stonerock et al., 1975), but results pointed to a faster passage rate in the cropectomized birds. Unfortunately, there was no information on dietary ingredients. Reduced phytate degradation as a consequence of lacking retention in the crop could have been confounded by dietary mineral P in regard to P retention. High Ca concentrations in the feed of laying hens could also have limited phytate hydrolysis in the intestine (the effect of Ca is discussed in detail in Chapter 3.3.4), and thus, the effect of cropectomy.

3.2.2 PROVENTRICULUS AND GIZZARD

In the proventriculus/gizzard, further "preparation" of the digesta for enzymatic digestion occurs. This is especially important for coarse dietary particles and digesta which bypasses the crop. Hydrochloric acid and pepsinogen are secreted by the proventriculus and mixed with digesta by muscular contraction in the gizzard; during contractions material is refluxed into the proventriculus (Svihus, 2014). The proventriculus and gizzard in this thesis were therefore considered as one compartment. Particles are ground and remain in the gizzard until broken

down to a critical size which can pass the pylorus (Svihus et al., 2002). Crushing can break down surrounding structures, and thus, increase accessibility of phytate from different feed fractions. Acid secretion may further extract phytate from the feed matrix. In this context, 21% of phytate from wheat bran was water-extractable and ball-milling of the bran to a mean particle size of 0.8 mm increased the proportion of water-extractable phytate up to 34% (Antoine et al., 2004). A further increase up to 49% was observed when the particle size was reduced below the mean aleurone cell dimensions (0.06 mm) (Antoine et al., 2004), which corresponds to the size of the majority of particles that enter the duodenum (<0.1 mm) (Hetland et al., 2002). Moreover, it was shown that exposure to pH 3 reduced the thickness of the aleurone cell wall from wheat and further caused the release of carbohydrates and Ca ions from the pericarp, and a small amount of carbohydrates, mainly polysaccharides, from aleurone layers (Morales et al., 2001). Such release can also be suggested to occur from the aleurone layer and other surrounding tissues of phytate in the acidic environment of the proventriculus/gizzard. Blaabjerg et al. (2010a) incubated feedstuffs with and without added phytase (size of the majority of particles: 1-3 mm) in water. The authors suggested that one part of the $InsP_6$ in different feedstuffs is readily degraded whereas another part is difficult to degrade. It was concluded that this is in part due to the presence of mineral-$InsP_6$ and/or protein-$InsP_6$ complexes that are rather resistant to hydrolysis, and/or because the $InsP_6$ is encapsulated in the cell wall matrix (Blaabjerg et al., 2010a). In the digestive tract of broilers, degradability of phytate is probably higher compared to degradability during the used incubation conditions *in vitro* due to the passage through the proventriculus/gizzard. It can be concluded from the discussed literature that very intense grinding (<0.1 mm) and acidification in the proventriculus/gizzard increase susceptibility and accessibility of dietary phytate in broilers. Proteolytic digestion in this segment may further increase phytate accessibility and solubility and effects can be even higher when digesta was soaked and enzymatically prepared in the crop.

$InsP_6$ hydrolysis in the proventriculus/gizzard was not calculated in the present work for the reasons described in Chapter 3.1. Most of the average pH values reported in the proventriculus/gizzard of broilers fed pelleted diets are between pH 3 and 4 (Svihus, 2011). This low pH favours protonation of the weak-acid groups of the $InsP_6$ molecule, which displaces minerals (Maenz, 2001). This means high solubility and thus degradability of most phytates in the proventriculus/gizzard. Microorganisms and fermentation have been reported in the gizzard (Józefiak et al., 2006; Rehman et al., 2007) so that low $InsP_6$ hydrolysis by acid-tolerant bacteria cannot be ruled out. It is also possible that phytases produced by

microorganisms in the crop are getting active during prolonged retention or under the prevailing conditions in the proventriculus/gizzard. In addition, intrinsic plant phytases (even if no phytase activity is detected in the diet), which are not extracted from the matrix of the diet using the protocols employed, may be extracted under the conditions of the proventriculus/gizzard. Subsequently, they may contribute to $InsP_6$ hydrolysis in the proventriculus/gizzard and intestinal segments. In the present work, concentrations of $InsP_6$ were significantly different in the crop but not in the proventriculus/gizzard between birds fed diets containing microwave treated and untreated wheat without supplemental phytase (Annex 2). This suggests that $InsP_6$ hydrolysis occurred in the proventriculus/gizzard of birds fed diets with low intrinsic phytase activity. Further $InsP_6$ hydrolysis in the proventriculus/gizzard by microbial and perhaps by intrinsic plant phytases could, in part, explain the high $InsP_6$ hydrolysis (55-67%) measured in the duodenum/jejunum of birds fed diets containing only low or no detectable phytase activity (Manuscript 1, 3 and 4). In contrast, Marounek et al. (2008) detected very low phytase activity in the digesta of the stomach of laying hens fed a maize-soybean meal based diet. Correspondingly, *Bifidobacteria* and *Lactobacilli* isolated from the crop and gizzard of broilers showed only a low phosphatase and phytase activity (Palacios et al., 2008). Hence, further studies are needed on phytase activity of different sources and $InsP_6$ hydrolysis to clarify the role of the proventriculus/gizzard in $InsP_6$ hydrolysis. However, the discussed findings demonstrate that an important role of the anterior segments is the enhancement of phytate accessibility for enzymes in the following segments when diets without detectable phytase activity are fed.

3.2.3 SMALL INTESTINE

Maximization of nutrient accessibility before digesta enters the duodenum is beneficial because retention time in the small intestine of poultry is short. Mean retention time of digesta recorded in the jejunum and ileum of broilers ranged between 136 min and 182 min (Weurding et al., 2001). Dänicke et al. (1999) reported a retention time of 8-18 min, 84-110 min, 104-140 min in the duodenum, jejunum and ileum of broilers respectively. In the present work, the majority of $InsP_6$ hydrolysis measured in the caeca occurred between the crop and duodenum/jejunum (55%; Manuscript 1) and continued in the ileum (16%, Manuscript 1) and caeca (19%; Manuscript 1) of broilers fed a maize-soybean meal-based diet without supplemental phytase or MCP. An $InsP_6$ hydrolysis of 62-74% (Manuscript 1-3) and 91% (Manuscript 1) was found in the lower ileum and caeca of broilers fed maize-soybean meal-based diets without detectable phytase activity. Correspondingly, P net absorption in the

lower ileum ranged between 52% and 60% (Manuscript 1-3). An $InsP_6$ hydrolysis of 70% and P retention of 48% was found based on excreta measurements (Manuscript 2). Other studies similarly found such high $InsP_6$ hydrolysis in broilers. Leytem et al. (2008) and Tamim and Angel (2003) reported an $InsP_6$ hydrolysis of 89% and 67% in the ileum of broilers fed low-Ca and low-P maize-based diets. An $InsP_6$ hydrolysis between 68% and 77% was detected in excreta of broilers fed maize-soybean meal based diets without supplemented phytase (Mohammed et al., 1991; Edwards Jr., 1993; Mitchell and Edwards Jr., 1996). These high levels of $InsP_6$ hydrolysis and P net absorption demonstrate that a great part of $InsP_6$-P can be available in broilers irrespective of dietary phytase activity. This contradicts the conventional view that $InsP_6$-P is poorly used by avian species because of insufficient endogenous $InsP_6$ hydrolysing enzymes (Adeola and Cowieson, 2011). Therefore, this long assumed conception of phytate hydrolysis being low in broilers needs revision.

The origin of phytases in broilers' digestive tract responsible for such high $InsP_6$ hydrolysis has been controversially discussed in the literature. Phytases originating from the mucosa and microbiota were suggested to be involved and will be discussed in the following subchapters.

Possible relevance of endogenous mucosal phytases

Chicken and rats appear to have higher endogenous mucosal phytase activity compared with humans and pigs (Lopez et al., 2002). In preparations of mucosa from the small intestine of broilers $InsP_6$-degrading activity has been detected *in vitro* (Biehl and Baker, 1997; Maenz and Classen, 1998; Abudabos et al., 2000; Applegate et al., 2003; Onyango and Adeola, 2009). Maenz and Classen (1998) reported that the phytase activity *in vitro* was highest for mucosa preparations of the duodenum and decreased progressively for preparations down the small intestine of broilers. This suggests that endogenous mucosal phytases play a major role in the anterior part of the small intestine. They could have contributed to the high $InsP_6$ hydrolysis measured in the duodenum/jejunum but also to further hydrolysis in the ileum when diets without detectable phytase activity were fed in the present work (Manuscript 1- 3). However, contribution of endogenous mucosal phytase activity to phytate hydrolysis in broilers is considered to be negligible in the prevailing literature. Only few studies led to the conclusion that endogenous mucosal phytase activity has a high capability to hydrolyse $InsP_6$ in the small intestine (Tamim and Angel, 2003; Tamim et al., 2004; Abudabos, 2012a,b).

Marounek et al. (2010) calculated a theoretical potential of mucosal phytase in laying hens to hydrolyse $InsP_6$. They sampled the total small intestine and detected a mucosal phytase activity (expressed as $InsP_6$ disappearance per g mucosa) of 11.5 µmol $InsP_6$/h. With a given

mucosal weight of 19.7 g the authors calculated a theoretical total mucosal activity of **3.8 µmol $InsP_6$/min** in the small intestine (Marounek et al., 2010). A similar calculation using results of the present work and the detected activity of Marounek et al. (2010) can give an approximation for the magnitude of $InsP_6$ hydrolysis in the digesta by mucosal phytase:

- For the calculation it was assumed that no $InsP_6$ hydrolysis occurred anterior to the duodenum.
- The generally accepted equation was used to calculate the maximal $InsP_6$ concentration which could enter the duodenum/jejunum (with the used maize-based diet from the present work) when no $InsP_6$ hydrolysis occurred anterior to this segment.
- $InsP_6$ hydrolysis was equated to zero and the Ti and $InsP_6$ concentrations which were detected in the diet and digesta from the duodenum/jejunum (Manuscript 1) were inserted in the equation:

$$0\%\ InsP_6\ \text{hydrolysis} = 100 - 100 \times \left(\frac{\text{Ti in the diet (3.2 g/kg DM)}}{\text{Ti in the digesta (6.5 g/kg DM)}}\right) \times \left(\frac{\mathbf{InsP_6\ in\ the\ digesta\ (\mu mol/g\ DM)?}}{InsP_6\ \text{in the diet (14.8 µmol/g DM)}}\right)$$

- The $InsP_6$ concentration in the digesta which entered the duodenum/jejunum when no $InsP_6$ hydrolysis occurred anterior to this segment was 30 µmol/g DM. Expressed per g of digesta (assuming a digesta DM concentration of 19% which was detected in the digesta from the duodenum/jejunum of broilers fed a pelletized wheat based diet (Engberg et al., 2004)): **5.7 µmol $InsP_6$/g digesta**.
- Assuming a digesta weight from the small intestine of 25 g, which was found by Marounek et al. (2010), 143 µmol $InsP_6$ were theoretically present in the digesta of the small intestine if no hydrolysis of feed $InsP_6$ occurred.

Thus, 3% of $InsP_6$ in the digesta of the small intestine could be hydrolysed by mucosal phytases per min (3.8 µmol $InsP_6$/min / 143 µmol $InsP_6$ × 100). In theory, taking into consideration a retention time of 150 min, mucosal phytase could hydrolyse the total amount of $InsP_6$ in the digesta, even if no hydrolysis occurs anterior to the duodenum/jejunum. However, these results have to be interpreted with great caution because these tissue preparations can contain other phosphatases, such as intracellular, intrinsic plant or microbiota-associated phytases. Results may further differ between laying hens and broilers as shown by Maenz and Classen (1998).

Studies using purified brush border membrane vesicles (BBV) from the jejunal mucosa of broilers also detected high phytase activity. The detected activity pointed to a higher potential phytase activity (expressed as P_i release from phytate per mg BBV per min) (up to **200 nmol P_i/mg BBV protein/min**) (Huber et al., 2015) compared to previous studies

using BBV (30 nmol P_i/mg BBV protein/min (Maenz and Classen, 1998)). Using these results from purified BBV the following theoretical magnitude of $InsP_6$ hydrolysis in the digesta by mucosal phosphatases was calculated:

- The average enrichment of apical membranes in BBV preparations compared to initial homogenates was about 2.04 (Huber et al., 2015). Taking this and the atomic weight of P (30.974 g/mol) into account a phytase activity (expressed as P_i release from phytate per mg BBV per min) of 3.0 µg P_i/mg mucosal homogenate protein/min can be calculated.
- Under the assumption of an average concentration of 38 mg protein/g mucosa homogenate (Yang et al., 2008) and a mucosa weight of the small intestine of 19.7 g (Marounek et al., 2010) a total mucosal phytase activity of **2.25 mg P_i/min** can be calculated.
- It was previously calculated that 143 µmol $InsP_6$ were theoretically present in the total digesta of the small intestine when no hydrolysis of feed $InsP_6$ occurred. The $InsP_6$ molecule contains 186 g P/mol. Hence, 26.6 mg of $InsP_6$-P were present in the total digesta of the small intestine.

From these calculations can be concluded that, in theory, 8% of $InsP_6$-P entering the small intestine can be liberated by enzymes from the mucosa of the small intestine per min (2.25 mg P_i/min / 26.6 mg $InsP_6$-P × 100). A retention time of 13 min would be sufficient to hydrolyse the feed $InsP_6$-P completely.

These calculations demonstrate the theoretical capability of mucosal phytases to hydrolyse a high proportion of $InsP_6$ from the digesta. However, calculated values cannot be extrapolated to *in vivo* processes. The calculations are based on several assumptions. It ignores prevailing conditions such as decreasing substrate and enzyme concentrations throughout the passage of the small intestine. In general, the activity and action of endogenous mucosal phytases on phytates in the digestive tract may differ from the activity and action on chemically synthesised phytate under *in vitro* conditions. Chemical conditions in the assay are optimised for endogenous mucosal phytase and ensure its maximal activity. Moreover, the localisation of endogenous mucosal phytases may restrict their contribution to luminal $InsP_6$ hydrolysis. Endogenous mucosal phytases are brush border membrane-associated enzymes (anchored in the apical plasma membrane of the microvilli as integral membrane proteins). It is unlikely that enzymes associated with desquamated cells are still active. The contribution of mucosal phytases to intraluminal $InsP_6$ hydrolysis therefore depends on the contact between $InsP_6$ from the digesta and phytases bound to the microclima of the unstirred water layer. This contact

may further be limited by digesta viscosity and retention time. But in contrast, reflux and muscular contractions may increase the exposure and contact of $InsP_6$ to endogenous mucosal phytases. Overall, it can be concluded that mucosal phytases are theoretically capable to hydrolyse high proportions of $InsP_6$ from the digesta. However, the magnitude of the contribution of phytases anchored in the apical membrane to luminal $InsP_6$ hydrolysis still needs clarification in further experimental works.

Using the data from the study of Huber et al. (2015) and own results, no significant correlation was found between endogenous mucosal phytase activity in the jejunum and $InsP_6$ hydrolysis in the duodenum/jejunum of broilers fed diets with or without MCP without added phytase. Similarly, Applegate et al. (2003) found no significant correlation between ileal $InsP_6$ hydrolysis and phytase activity (V_{max}) within BBV prepared from small intestinal mucosa of broilers from different strains fed diets with varying Ca concentrations. Correlation between ileal $InsP_6$ hydrolysis and phytase activity also was not significant when diets with or without 25-hydroxycholecalciferol and varying dietary Ca concentrations were fed (Applegate et al., 2003). However, ileal $InsP_6$ hydrolysis and endogenous mucosal phytase activity correlated significantly ($r=0.31$; $P \leq 0.03$) when different Ca sources and different 25-hydroxycholecalciferol and Ca concentrations were used (Applegate et al., 2003). These results taken together indicate that endogenous mucosal phytase is not the only phytase source responsible for $InsP_6$ hydrolysis in the small intestine.

Contribution of microbiota-associated phytases

It was suggested that phytate is partly hydrolysed by phytases produced by microorganisms present in the intestine of broilers (Kerr et al., 2000; Leytem et al., 2007; Leytem et al., 2008). In rats, it was already demonstrated that $InsP_6$ hydrolysis almost fails to occur when animals are germfree (Savage et al., 1981; Wise and Gilburt, 1982). $InsP_6$-degrading activity has been described for various bacteria *in vitro* (Konietzny and Greiner, 2002; Vats and Banerjee, 2004). Raghavendra and Halami (2009) reported a potential of different lactic acid bacteria isolated from chicken intestine to hydrolyse commercially available $InsP_6$ under specific conditions. In line with this observation, Angel et al. (2005) found an increased P retention in broilers when various *Lactobacillus* species were added to the diet. This indicates that bacteria such as *Lactobacilli* are capable to break down phytate within the digestive tract. *Lactobacilli* represent a high proportion of bacteria in the small intestine of broilers (Rehman et al., 2007). So bacterial phytase activity in the small intestine can also explain the recently

more often reported high $InsP_6$ hydrolysis in the small intestine or excreta of broilers fed diets without detectable phytase activity.

Marounek et al. (2008) incubated sodium phytate with digesta from the small intestine of laying hens fed a maize-soybean meal-based diet and detected considerable $InsP_6$-hydrolysing activity (14 µmol $InsP_6$/g digesta/h). Digesta weight from the small intestine was 16 g (Marounek et al., 2008), which results in a total phytase activity in the digesta of the small intestine of 3.7 µmol $InsP_6$/min. Using the previously calculated $InsP_6$ concentration (5.7 µmol/g digesta) which may enter the duodenum/jejunum, phytase in the total digesta was theoretically able to hydrolyse 4.0% of total $InsP_6$ in the small intestine per min. In theory, taking into consideration a retention time of 150 min, phytase in the digesta could hydrolyse the total amount of $InsP_6$ in the digesta of the small intestine. As discussed previously, it is unlikely that mucosal-linked phytase activity contributed considerably to the phytase activity measured in the digesta. This suggests that the reported phytase activity in the digesta was primarily of microbial origin. Thus, apart from endogenous mucosal phytases microbiota-associated phytases can be active and responsible for $InsP_6$ hydrolysis in the small intestine. However, the potential to hydrolyse feed phytate under the conditions of the digestive tract may differ and still needs to be clarified.

The much higher phytase activity and $InsP_6$ hydrolysis in the digesta from the small intestine compared to the crop reported in the present work and by Marounek et al. (2008) may be related to differences in the composition of the microbial population and in the quantity of specific bacteria in these segments. An accompanying study to the present work (Manuscript 3) identified *Lactobacilli* as the dominating microorganisms in the crop, jejunum and ileum of broilers fed a maize-soybean meal-based diet irrespective of MCP or phytase addition (Camarinha-Silva et al., 2015). However, the authors reported differences in the microbial clusters and the phylotypes of *Lactobacilli* between digesta from the crop and jejunum/ileum (Camarinha-Silva et al., 2015). In accordance, *Bifidobacteria* and *Lactobacilli* isolated from the small and large intestine showed higher phosphatase and phytase activities than those isolated from the crop and gizzard (Palacios et al., 2008). Further, genes coding phytase have been found in the genomes of *Bif. longum* subsp. *infantis* and *Bif. pseudocatenulatum*, whereas they were absent in most of the remaining investigated *Bifidobacteria* species (Tamayo-Ramos et al., 2012). This indicates that differences in composition of the microbial population and in species composition within a bacterial genus in part were crucial for the reported differences in phytase activity and phytate hydrolysis between the crop and small

intestine. Further studies are needed which differentiate between phytase and non-phytase producing bacteria in the digesta of different segments and quantify these bacteria.

3.2.4 HINDGUT

Rehman et al. (2007) investigated the bacterial population in different segments of the broiler digestive tract and found highest bacterial density and diversity in the caeca. Marounek et al. (2008) detected the highest phytase activity in digesta from the caeca compared to digesta from the other segments of the digestive tract of laying hens fed a maize-soybean meal-based diet. Correspondingly, concentrations of $InsP_6$ were higher in the caeca of gnotobiotic broilers compared to conventional broilers (Kerr et al., 2000). This points to an important impact of hindgut microorganisms on $InsP_6$ hydrolysis which was confirmed by the very high $InsP_6$ hydrolysis determined in the caeca (>90%) (Manuscript 1). An accompanying study to the present work showed that the caeca were predominantly colonised with *Bacteroidaceae*, *Ruminococcaceae* and uncultured *Clostridiales* (Camarinha-Silva et al., 2015). Since specific *Bacteroidaceae*, *Ruminococcaceae* and *Clostridiales* strains were shown to grow on phytate (Yanke et al., 1998; Steer et al., 2004), species from all three genera were probably involved in $InsP_6$ hydrolysis in the caeca in the present work.

Only a small proportion of the ileal digesta enters the caeca (Son et al., 2002). Thus, at a high level of $InsP_6$ hydrolysis in the ileum (74%, Manuscript 1), hydrolysis in the caeca is quantitatively less important. The subsequent passage through the rectum is unlikely to increase $InsP_6$ hydrolysis substantially due to the short retention time (23-36 min according to Dänicke et al., 1999). This is supported by the findings of Leytem et al. (2008), who showed no differences between $InsP_6$ hydrolysis measured in the ileum (89%) and excreta (90%) of broilers fed a maize-based diet. In contrast, Kerr et al. (2000) manifested their hypothesis of a high importance of hindgut microorganisms in $InsP_6$ hydrolysis by the comparison of ileal and fecal $InsP_6$ hydrolysis. But there was no information about the measured levels of $InsP_6$ hydrolysis. However, P absorption posterior to the upper ileum is not meaningful (Hurwitz and Bar, 1970; Shastak et al., 2012). Hence, the contribution of InsP-P liberated in the hindgut to the P supply of poultry is negligibly low. This was confirmed by similar tibia ash concentration and total tibia ash of cecectomized and sham-operated broilers fed diets without added phytase (assuming that bacteria in sham-operated birds were able to hydrolyse $InsP_6$) (Biehl and Baker, 1997).

It can be concluded that the frequently reported high $InsP_6$ hydrolysis and related P net absorption in broilers fed diets without detectable phytase activity is predominantly caused by

the activity of endogenous mucosal and microbiota-associated phytases in the small intestine. A contribution of phytases which are active in the proventriculus/gizzard cannot be ruled out.

3.3 FACTORS AFFECTING $InsP_6$ HYDROLYSIS

3.3.1 EFFECT OF MICROWAVE TREATMENT

Heat treatment of grains such as wheat or barley can reduce intrinsic phytase activity (Carlson and Poulsen, 2003; Blaabjerg et al., 2010a), which agrees with results in Manuscript 4. Reduction of intrinsic plant phytase activity by heat treatment of cereals was reported to reduce $InsP_6$ hydrolysis in the stomach and ileum of pigs (Schlemmer et al., 2001; Blaabjerg et al., 2010b). Lower $InsP_6$ hydrolysis was also shown in heat-treated compared to non-heat-treated wheat during incubation for 2 h at 20°C *in vitro* (Blaabjerg et al., 2010a). Correspondingly, Carlson and Poulsen (2003) found lower $InsP_6$ hydrolysis when heat-treated compared to non-heat-treated wheat- or barley-based diets were incubated in water for different times up to 24 h at 38°C. In agreement with these findings, the present work showed a significantly lower $InsP_6$ hydrolysis in the crop of birds fed the basal diet containing microwave treated wheat (26%) instead of untreated wheat (59%) without added phytase (Manuscript 5). This was most likely the consequence of reduced activities of wheat phytases and enzymes which are responsible for the breakdown of structures surrounding phytate due to microwave treatment.

Microwave treatment may also cause other changes. Grain processing provides increased exposure of substrates to enzymes in the digestive tract by disrupting the seed coat and cell walls, and reducing particle size (Amerah et al., 2011). Heat treatment may induce the breakdown of chemical bonds and denaturation of proteins (Björck and Asp, 1983). Correspondingly, microwave treatment and associated heat exposure may induce changes in binding forms of phytate and in structures of surrounding tissues, which may change accessibility and degradation of phytate by phytases. In agreement with this, Blaabjerg et al. (2010a) revealed a higher $InsP_6$ hydrolysis in heat-treated compared to non-heat-treated wheat after 24 h of soaking *in vitro*. Similarly, heat-treatment of wheat increased the relative instantaneously degradable fraction of phytate during incubation of wheat (Blaabjerg et al., 2012). However, the present work indicated that in the crop of broilers, reduction in intrinsic phytase activity probably overweighted such other effects of microwave treatment in respect to $InsP_6$ hydrolysis (Manuscript 5). But this seems to change in the intestine of broilers. $InsP_6$ hydrolysis was similar in the duodenum/jejunum and even higher in the ileum of birds fed the

basal diet containing microwave treated wheat (78%) instead of untreated wheat (69%) without supplemental phytase (Manuscript 4). Different reasons for these results were discussed in Manuscript 4: A higher activity of microbiota-associated and endogenous mucosal phytases in the small intestine of birds fed the basal diet containing microwave treated wheat instead of untreated wheat was assumed; higher phytase activity may be related:

1. with the aforementioned presence of the more accessible substrate in the basal diet containing microwave treated wheat; 2. with increased enzyme expression induced by higher amounts of available substrate in birds fed the basal diet containing microwave treated wheat instead of untreated wheat; and 3. increased accessibility of other nutrients, transformation of chemical structures, composition and configuration of nutrients, increased fibre solubility or digesta viscosity by microwave treatment might have affected microbial composition shifting towards more phytase-producing organisms.

The aspect of digesta viscosity is now discussed in more detail. Processing of feed at high temperatures is known to increase intestinal digesta viscosity (Amerah et al., 2011), in part by increasing starch gelatinisation and fibre solubility (Østergård et al., 1989; Gracia et al., 2003; Svihus, 2006). A conditioning temperature above 80°C (Cowieson et al., 2005) or heat treatment in an autoclave at 100°C for 5 min (Svihus et al., 2000) increased viscosity of the diet. In the present work, the maximum temperature measured in wheat meal after 6 min of microwave treatment was 106°C, which suggests that microwave and the related heat treatment also increased viscosity of diet and digesta here. Increased digesta viscosity was shown to increase microbial activity in the digestive tract of broilers (Carré et al., 1995; Choct et al., 1996). In addition, there is an association between higher ileal viscosities and slower rate of passage of Fe_2O_3 (Sacranie et al., 2012a). It can thus be concluded that higher retention time of digesta in the intestine and perhaps higher amounts of phytase-producing microorganisms caused by increased digesta viscosity contributed to high $InsP_6$ hydrolysis in the small intestine of birds fed the basal diet containing microwave treated wheat (Manuscript 4). In general, increased digesta viscosity is described to reduce the contact between enzyme and substrate. However, heat processing of barley increased digesta viscosity and villus height in the jejunum of broilers (Gracia et al., 2003). Perhaps villus height further facilitated the access of endogenous mucosal phytases to phytate in the digesta of birds fed the basal diet containing microwave treated wheat (Manuscript 4).

Although microwave treatment of wheat increased $InsP_6$ hydrolysis in the ileum of broilers, the benefit of application of microwaves is questionable. This processing implies negative

nutritional aspects such as the loss of endogenous enzymes, vitamins and available nutrients, such as proteins, free amino acids or starch, e.g. through Maillard complexing (Björck and Asp, 1983). Further, the missing increase in P net absorption by microwave treatment despite of the increase in $InsP_6$ hydrolysis in the ileum (Manuscript 4) points to a lacking benefit in P excretion. Hence, microwave treatment of wheat will not help regarding P net absorption and reduction of P excretion of broilers.

3.3.2 CONTRIBUTION OF INTRINSIC PLANT PHYTASE

Substantial intrinsic phytase activity has been detected in different plant feedstuffs (Eeckhout and De Paepe, 1994; Viveros et al., 2000; Steiner et al., 2007) and conditions in the crop may promote intrinsic phytase activity. Nevertheless, until recently, the significance and potential of intrinsic plant phytase to hydrolyse phytate in the digestive tract of broilers attracted little attention. Therefore, this subchapter discusses the contribution and efficacy of intrinsic plant phytase in the digestive tract of broilers.

Crop

The high $InsP_6$ hydrolysis (59%) in the crop of broilers fed the basal diet containing untreated wheat without supplemental phytase demonstrates that wheat phytase is able to act on phytate and incubation conditions in the crop are appropriate for wheat phytase to be active (Manuscript 5). This supports *in vitro* properties of phytase from wheat: The pH measured in the crop of broilers fed this diet (5.3+/-0.4, data not shown) was close to the *in vitro* determined pH optimum of phytases from wheat (phytase 1: 6.0 and phytase 2: 5.5) (Nakano et al., 1999); at approximate body temperature of broilers, purified wheat phytase is generally stable and still shows an activity of 80-90% of the *in vitro* analysed temperature optimum (Nakano et al., 1999); this was confirmed for in-feed plant phytase by a faster hydrolysis of comparable amounts of $InsP_6$ when wheat-based diets were soaked in water at 38°C compared to 20°C (Carlson and Poulsen, 2003). Hydration and the initiating enzymatic degradation of the feed matrix in the crop probably extract water-extractable phytate and promote the contact between phytase and phytate. High $InsP_6$ hydrolysis in a wheat-based diet found in the crop (Manuscript 5) agrees with hydrolysis of $InsP_6$ during soaking of cereal meal-based diets in water at temperatures between 10°C and 38°C (Skoglund et al., 1997; Carlson and Poulsen, 2003; Lyberg et al., 2005; Lyberg et al., 2006; Blaabjerg et al., 2010a). It further shows that a great proportion of phytate from wheat and soybean meal can be soluble and accessible for wheat phytase under the prevailing conditions in the crop. In the present work, retention time allowed for more than half of feed $InsP_6$ to be hydrolysed, which corresponds to values found

for $InsP_6$ hydrolysis in the crop of broilers fed maize-based diets supplemented with 500 FTU/kg of microbial phytase (Manuscript 5). This clearly illustrates the high potential of intrinsic wheat phytase to hydrolyse $InsP_6$ in the crop. Thus, intrinsic plant phytase can make a high contribution to the total $InsP_6$ hydrolysis in the digestive tract of broilers.

It can be speculated that an increase in the efficacy of intrinsic plant phytase in the crop still is possible. Strategies that manipulate retention time in the crop and the use of the crop may affect the action of intrinsic phytases. Feeding coarsely ground or whole cereal grains instead of ground cereal grains can increase the volume of digesta in the gizzard which could increase retention time in the gizzard (Svihus et al., 2002), and thus, in the crop. However, coarse particles may impair the contact between phytate and phytases in the crop where wheat phytase is predominantly active. It is unlikely that longer retention in the crop and the reflux of ground particles from proventriculus/gizzard are sufficient to compensate for this. A more promising approach could be intermittent feeding, which can stimulate birds to use the crop as a storage organ and thus can increase retention time (Svihus et al., 2010; Svihus, 2014). As discussed later in more detail, ground feed could promote accessibility and thus hydrolysis of phytate by wheat phytase in the crop, but addition of structural components is necessary for development of a functioning gizzard. Alternatively, addition of enzymes hydrolyzing surrounding structures of phytate and phytase could increase the action of wheat phytase. In the present work, it was shown that addition of xylanase alone did not increase $InsP_6$ hydrolysis in the crop of birds fed the basal diet containing untreated wheat (Manuscript 5). But the addition of enzyme cocktails which hydrolyse different structures may be more effective and could increase $InsP_6$ hydrolysis by intrinsic wheat phytase.

In addition to intermittent feeding, the use of diets with high intrinsic enzyme activity may compensate for the short retention time in the crop. In this context, it was shown that phytase activities of 121 U/kg and 632 U/kg of diet (phytase activity originated from wheat) resulted in an $InsP_6$ hydrolysis of 26% and 59% in the crop (Manuscript 5). The use of diets with intrinsic phytase activity even higher than those in the diets used in the present work could further increase $InsP_6$ hydrolysis in the crop. Classical plant breeding towards high phytase crops may be one strategy to realize high intrinsic plant phytase activity. However, selection of cultivars with varying intrinsic phytase activity is related to differences in other dietary factors, such as concentrations of Ca, P or NSP (Kluge and Dusel, 2004; Steiner et al., 2007), which may affect the action of intrinsic phytase. Other differences in structures and binding forms may affect accessibility and solubility of phytate and thus its hydrolysis in the gut.

In vitro, P_i was already shown to be an inhibitor of purified plant phytase activity (Greiner et al., 2000b). Addition of calcium carbonate reduced $InsP_6$ hydrolysis during soaking of a barley-based diet (Larsen et al., 1999). Better knowledge is needed about these interactions to find a cultivar realizing high $InsP_6$ hydrolysis by intrinsic phytase in the digestive tract. The modification of gene activity/expression by genetic engineering may be a tool to increase intrinsic plant phytase activity. Moreover, the introduction of genes encoding specific phytases in cereals may increase $InsP_6$ hydrolysis by "phytases from plant feedstuffs" in broilers (Brinch-Pederson et al., 2002). It was shown that growth rates of broilers were higher when diets containing transgenic seeds with constructs compromising a phytase gene from *A. niger* were fed compared to diets with non-transgenic seeds (Pen et al., 1993). Further, the efficacy of microbial phytase expressed in canola and in *Aspergillus* was similar for enhancing the utilization of phytate P in broilers fed corn-soybean meal-based diets (Zhang et al., 2000).

Proventriculus/gizzard

As already introduced, conditions in the gizzard are generally reported to be detrimental to intrinsic plant phytases. However, following incubation at pH 3.5 in the presence of 5 mg/ml pepsin 70% of the original purified wheat phytase activity was still recovered; Activity slightly decreased at pH 3 and was lost at pH 2.5 (Phillippy, 1999). Average pH values in the proventriculus/gizzard of poultry range between 3.0 and 4.0 (Svihus, 2010). Phytase protected by the surrounding feed matrix is probably even more stable than the purified assayed form. Hence, activity of wheat phytases in the proventriculus/gizzard cannot be ruled out. The previously discussed effect of grinding and acid secretion in the proventriculus/gizzard promoting solubility and accessibility of phytate may further have increased the action of intrinsic phytase in the stomach and the intestine.

In regard to the pattern of lower InsPs, the $InsP_4$ and $InsP_5$ isomer which were predominantly formed by wheat phytase in the crop dominated in the proventriculus/gizzard of birds fed the basal diets containing untreated wheat without added phytase (Annex 2). This is an indication for the activity of intrinsic wheat phytase in the proventriculus/gizzard to proceed. Multiple forms of phytases exist in cereals as described for wheat and lupin (Lim and Tate, 1973; Konietzny and Greiner, 2002). In *in vitro* stability studies, determined properties only apply to the specific purified enzymes. Other isoenzymes in wheat may show different properties and activity under the conditions of the proventriculus/gizzard.

Small intestine

Passing the proventriculus/gizzard without irreversible inactivation, isoenzymes of intrinsic plant phytase may be active in the small intestine. In the small intestine of poultry the average pH values range between 6.5 and 7.5 (Svihus, 2010). Activity of purified wheat phytases was stable at pH 7 (Nakano et al., 2000) and most plant phytase activities dramatically decrease at pH values just above 7.5 (Konietzny and Greiner, 2002). The existence of an alkaline plant phytase was already described in *Lily pollen* (Barrientos et al., 1994). In addition, following incubation with 2.5 mg/ml pancreatin 60% of purified wheat phytase activity still remained (Phillippy, 1999). Therefore, further activity of phytases from wheat meal and contribution to $InsP_6$ hydrolysis even in the small intestine is possible. However, further experimental work is required to differentiate between the contribution of phytases from wheat and of other origin in different segments of the digestive tract.

In literature, results on the benefit of intrinsic plant phytase to intestinal P digestibility in poultry are inconsistent. Few studies using different grain varieties found a linear relationship (Barrier-Guillot et al., 1996) or significant positive correlation between the level of intrinsic grain phytase activity and P retention in broilers (Oloffs et al., 2000). In these studies, P retention of broilers fed wheat-based diets ranged between 45% and 70% (64-775 U/kg phytase) (Barrier-Guillot et al., 1996) and between 36% and 57% (510-1,249 U/kg phytase) (Oloffs et al., 2000). In another study, P retention in broilers was low when a maize-soybean meal-based diet was fed (35%) and significantly higher when wheat bran and wheat were added (50%), probably due to intrinsic wheat phytase activity (Paik, 2003). Oloffs et al. (1998) used different proportions of extruded and unextruded wheat and found significantly higher P retention in broilers fed diets containing 75% unextruded wheat (44%) (830 U/kg of phytase) than for those containing 100% extruded wheat (25%) (without detectable phytase activity). In contrast, other studies did not find any dependence between the level of intrinsic phytase and phytate hydrolysis in broilers (Nelson, 1976, Juanpere et al., 2004, Leytem et al., 2008). In consistence with the latter results $InsP_6$ hydrolysis in the duodenum/jejunum and ileum in the present work was not significantly higher with high compared to low dietary intrinsic phytase activity (Manuscript 2 and 4). In the present work and the studies of Leytem et al. (2008) and Juanpere et al. (2004), endogenous mucosal and microbiota-associated phytases probably had a higher influence than intrinsic phytases. $InsP_6$ hydrolysis and P digestibility in those studies was at a high level even for the diets with low or non-detectable phytase activity ($InsP_6$ hydrolysis: 62-89%; P digestibility: 54-86%). Accessibility and solubility of phytate and endogenous mucosal and microbiota-associated phytase activity

were obviously high enough and other conditions promoted phytase action so that those phytases could compensate for the low intrinsic phytase activity. In contrast, in studies which showed an effect of intrinsic phytase activity, P retention values found for diets with low intrinsic phytase activity were at a low level (Barrier-Guillot et al., 1996, Oloffs et al., 2000; Paik, 2003). In these studies hydrolysis by plant phytases which predominantly occurs in the crop was probably higher than hydrolysis that was possible in the small intestine by endogenous mucosal and microbiota-associated phytases. Hence, intrinsic phytase activity may have a beneficial effect on overall $InsP_6$ hydrolysis in broilers e.g. when diets contain mineral P or high levels of Ca which may reduce, as discussed later in more detail, $InsP_6$ hydrolysis in the intestine. Correspondingly, a high dietary Ca:$InsP_6$ ratio caused by high Ca concentrations (8.1 g/kg of diet, 7.4 g/kg of DM and 45-54 g/kg of DM) could have caused low P retention for diets without or with low phytase activity (Barrier-Guillot et al., 1996; Oloffs et al., 1998; Oloffs et al., 2000). Diets in the study of Barrier-Guillot (1996) additionally contained mineral P. It can be concluded from the present work and related literature that intrinsic plant phytase cannot increase the overall phytate hydrolysis in broilers which have a high capacity to hydrolyse phytate in the gut. However, it can be speculated that the previously discussed strategies to increase the action and activity of intrinsic plant phytase in the crop may result in $InsP_6$ hydrolysis exceeding those which occurred in the crop of birds from the present work and discussed literature. Then the achieved level of $InsP_6$ hydrolysis in the crop could exceed the potential of mucosal or microbiota-associated phytases in the small intestine even if those are highly capable to hydrolyse $InsP_6$. However, this speculation needs to be proven by experimental works.

In the aforementioned studies and in the present work differences in intrinsic phytase activities were achieved by feed processing or the use of different grain species or cultivars. Related other variation than differences in intrinsic phytase activity e.g. in nutrients or structures may further have affected $InsP_6$ hydrolysis as previously discussed for microwave treatment (Chapter 3.3.1). Thus, these approaches are not suitable to evaluate the relevance of dietary intrinsic phytase activity for total $InsP_6$ hydrolysis in the digestive tract of broilers. To avoid confounding factors different doses of purified wheat phytase could be added to a basal diet. However, purified phytase most likely differs in properties and performance in the digestive tract compared to phytase enclosed in the feed matrix. To study the sole influence of intrinsic phytase in plant feedstuffs under approximate ceteris paribus conditions the use of genetically engineered cereals for increased phytase activity seems to be necessary.

3.3.3 EFFECT OF SUPPLEMENTED MICROBIAL PHYTASE

The previous chapters showed the high potential of microbiota-associated, endogenous mucosal and intrinsic plant phytases to hydrolyse $InsP_6$ and that further improvement in $InsP_6$ hydrolysis in the digestive tract of broilers is possible. Therefore, the capability of microbial phytase supplements to hydrolyse $InsP_6$ in different segments of the digestive tract of broilers is addressed in this subchapter.

Results of the present work agree with the general assumption from literature that the anterior parts of the digestive tract are the primary sites of action of added phytase in broilers (Elkhalil et al., 2007; Greiner, 2010). High $InsP_6$ hydrolysis in the presence of *E. coli* or *Aspergillus* derived phytases in the crop (up to 70% with 500 FTU/kg and 80% with 12,500 FTU/kg phytase) (Manuscript 1 and 5) demonstrates that phytate from maize and soybean meal was highly soluble and accessible for supplemented phytase in this segment. Additional $InsP_6$ hydrolysis measured in the duodenum/jejunum (Manuscript 1, 3, 4 and 5) was assumed to be primarily caused by the action of added phytase in the proventriculus/gizzard due to a more favourable pH range than in the small intestine (Manuscript 1). In addition, high solubility of phytate promotes the activity of added phytase in the stomach. The InsP pattern found in the proventriculus/gizzard indicated activity of added phytase in this segment (Manuscript 1; Annex 2 and 3). In accordance, a higher activity of supplemented phytase was detected in the crop and proventriculus/gizzard compared to the small intestine (Yu et al., 2004). Leslie et al. (2006) investigated the effect of supplemented phytase on InsPs in different segments of the digestive tract of broilers and reported that the effect of supplemented phytase on the amounts of $InsP_6$ was primarily seen in the crop and proventriculus/gizzard. When in the present work diets supplemented with 500 FTU/kg phytase were fed, additional $InsP_6$ hydrolysis occurred between the duodenum/jejunum and ileum (up to 14 percentage points) (Manuscript 1, 3 and 4). This was assumed to be in part related to residual activity of supplemented phytase in these segments. The InsP pattern found in the duodenum/jejunum and ileum indicated activity of added phytase in these segments (Manuscript 1, 3 and 4). Correspondingly, residual phytase activity was detected in the duodenum, jejunum (Yu et al., 2004; Onyango et al., 2005) and ileum of broilers fed diets supplemented with an *E. coli* or *P. lycii* derived phytase (Onyango et al., 2005) and after incubation of *Aspergillus*, *Peniophora*, *E. coli*, *Klebsiella* or *Bacillus* derived phytase in digesta supernatants from those segments (Igbasan et al., 2000; Elkhalil et al., 2007). In contrast, Liebert et al. (1993) detected no phytase activity in the digesta of the small intestine of broilers fed diets supplemented with an *A. niger* derived phytase. Yu et al. (2004) found no

phytase protein in the ileal digesta of broilers fed diets supplemented with a *P. lycii* derived phytase. In accordance with this, higher phytase activity was shown with an *E. coli* compared to an *P. lycii* derived phytase in all sections of the digestive tract of broilers (in FTU per kg DM intake) (Onyango et al., 2005). Further, two out of three *Aspergillus* derived phytases showed lower residual activity compared to *Peniophora*, *E. coli*, and *Bacillus* derived phytases after incubation in digesta from the jejunum and ileum (Igbasan et al., 2000). Varying activity in the small intestine may be related to differences in the methods for determination of phytase activity. But it is also well-known that different phytase sources differ in their performance in the digestive tract due to the already introduced differences in properties such as resistance against proteolytic degradation or activity at prevailing pH. Higher stability against pancreatin was found for phytase from *E. coli* and *Bacillus* compared to *Peniophora* and *Aspergillus* derived phytase (Igbasan et al., 2000) and for phytase from *E. coli*, *Klebsiella* and *Bacillus* compared to *Aspergillus* derived phytase (Elkhalil et al., 2007). From those enzymes *E. coli* and *Klebsiella* derived phytase showed the highest resistance against pepsin (Igbasan et al., 2000; Elkhalil et al., 2007). These findings can, in part, explain the described differences in activity of different phytases in the small intestine. Overall, results from the present work and related literature demonstrate that added phytase is primarily active in the crop and proventriculus/gizzard. Residual activity in the small intestine depends on the properties of the used phytase. However, results from the present work and literature cannot separate the contribution of microbiota-associated, endogenous mucosal and supplemental phosphatases to $InsP_6$ hydrolysis in the digestive tract. Further experimental work is needed that investigates the microbial population, endogenous mucosal phosphatases and activity of supplemental phytase (probably by specific Enzyme Linked Immunosorbent Assay).

The differences in $InsP_6$ hydrolysis between the experiments conducted within this thesis in the crop of birds fed maize-soybean meal-based diets supplemented with the same *E. coli* derived phytase (Manuscript 1: 44%; Manuscript 5: 60%) may in part be related to the analyzed dietary phytase activity (Manuscript 1: 442 FTU/kg; Manuscript 3: 620 FTU/kg; intended activity was 500 FTU/kg in both experiments). The differences found in $InsP_6$ hydrolysis between the different supplemented phytases in the crop and proventriculus/gizzard in the present work can be related to differences in properties such as enzyme kinetics, pH, susceptibility to the electrostatic environment in the stomach or resistance against gastrointestinal proteases as discussed in Manuscript 1. Another explanation can be the assay conditions for determination of phytase activity for the included dose of

phytase in the diets. The analyzed activity in the diets containing different phytases was at the same level. However, activity was determined under the more favourable conditions of the respective phytase product (*Aspergillus* phytase: assay at pH 5.5 and 37°C; *E. coli* phytase: assay at pH 4.5 and 60°C). At an approximate body temperature of 42°C in chicken those phytases can show different activities which was already discussed in Manuscript 1. This can also explain the differences in $InsP_6$ hydrolysis between diets supplemented with different phytases in the crop (Manuscript 1). Thus, in studies comparing the efficacy of different phytases in broilers the included activity should be similar at 42°C. In addition, it was reported that the differences in activity (relative to the maximal activity at 37°C and optimal pH) of microbial phytases vary in dependence on pH, even in the range of pH 4-6 (Elkhalil et al., 2007; Brüning, 2009). Since pH in the crop and proventriculus/gizzard varies and cannot be predicted, pH in the assay should reflect the pH optimum of the respective phytase. Moreover, sodium phytate or other commercially available phytates are generally used as substrate for determination of phytase activity. Activity of different phytases on naturally occurring phytate within the feed matrix and diet can differ from the pure standards. Tran et al. (2011) compared the relative activity (pH 3, 37°C) of four commercial phytases using $InsP_6$ from $InsP_6$-soy protein, $InsP_6$-lysozyme complex or sodium phytate. Activity differed between the phytases and depended on the used substrate. Differences in activity between the phytases were higher when $InsP_6$-soy protein or $InsP_6$-lysozyme was used as substrate compared to sodium phytate. Bohn et al. (2007) demonstrated that the use of the naturally occurring phytate globoids as substrate for wheat phytase slowed the action of the enzyme compared to using commercially available phytate as substrate. As suggested by Selle and Ravindran (2007) the assay could be based on a substrate other than commercially available phytate. In experiments, diets which are supplemented with phytases contain ingredients which may influence phytate hydrolysis by different phytases to a varying extent. Hence, the use of the respective mixed diet in the assay could also be beneficial. Overall, it can be concluded that differences in efficacy of different phytases found within an experiment or between different experiments in literature are in part related to varying properties in different segments of the digestive tract. Furthermore, they may be related to the assay conditions for determination of phytase activity, when specific conditions differ for different phytase supplements. Differences in these conditions between different experiments using the same phytase may also contribute to differences in efficacy of the same phytase reported in the literature. In efficacy comparisons of different phytases, these conditions should be reconsidered to assure that differences in efficacy are primarily related to different enzyme

performance under the conditions of the digestive tract. At least, a temperature of 42°C and a pH reflecting the optimum of the included phytase should be adjusted.

In the present work, a very high dose of supplemented phytase (12,500 FTU/kg) increased $InsP_6$ hydrolysis in the small intestine up to more than 90%; 80% of hydrolysis already occurred in the crop (Manuscript 3 and 5). This confirms that a high activity of phytase in the anterior segments is favourable for high $InsP_6$ hydrolysis in the digestive tract due to precipitation and less favourable conditions in the small intestine. Since no further $InsP_6$ hydrolysis occurred between the duodenum/jejunum and ileum, a complete hydrolysis of feed $InsP_6$ seems not to be possible and limited by accessibility or solubility of phytate or retention time. In the present work and literature high doses of phytase caused enhancements in degradation of $InsP_6$ and lower InsPs, pc P net absorption, P retention and performance (Manuscript 3 and 5). However, more recent reviews, especially in human sector, emphasize the beneficial metabolic effects of dietary $InsP_6$ (Konietzny et al., 2006; Harland and Morris, 1995; Schlemmer et al., 2009; Ali et al., 2010; Kumar et al., 2010). In brief, $InsP_6$ functions as a natural antioxidant due to its potential to complex iron. This prevents the formation of highly reactive hydroxyl radicals (Schlemmer et al., 2009). As diseases such as neurodegenerative diseases or cirrhosis have been linked with radicals, $InsP_6$ was suggested to have a preventive role (Schlemmer et al., 2009). $InsP_6$ and $InsP_5$ are involved in neurotransmission and can modulate the regulation of exo- and endocytosis processes (Sasakawa et al., 1995; Efanov et al., 1997). Dietary phytate has shown to reduce serum lipid and cholesterol levels (Jariwalla, 1999; Onomi et al., 2004) and was reported as anticarcinogen, as preventive tool against diabetes mellitus, coronary heart disease, renal stone incidence (Konietzny et al., 2006) and fatty liver (Katayama, 1999). Since lower InsPs and *myo*-inositol are also involved in metabolic processes (as discussed later in more detail), in part, the effects of dietary phytate may be related to its degradation products. Thus, very high disappearance of $InsP_6$ and lower InsPs in the intestinal digesta due to high doses of supplemented phytase may not only have beneficial effects. However, the relevance of positive metabolic effects of $InsP_6$ and lower InsPs in poultry and for poultry production is still unclear.

3.3.4 Effect of mineral P and Ca

In the present work and literature phytate-P utilization in broilers fed diets without added phytase was reported to range between 0% and 90% (Waldroup et al., 1965; Nelson, 1967; Nelson, 1976; Mohammed et al., 1991; Edwards Jr., 1993; Mitchell and Edwards Jr., 1996;

Tamim and Angel, 2003; Leytem et al., 2008). It was previously discussed (Chapter 3.2) that the frequently reported low phytate hydrolysis is not related to insufficient compatible enzymes in broilers. In the literature, differences in efficacy of phytase between studies were frequently reported. These inconsistencies may be related to different factors which have been discussed to affect the activity and efficacy of phytases in the gut. Dietary constituents which can reduce phytate hydrolysis are mineral P and Ca. In the common practice poultry feeds contain mineral P and Ca to cover the birds' requirement. Mineral P is included regardless of the utilization of phytate-P to achieve and exceed the recommended levels of non-phytate P. Knowledge about the interactions between mineral P and Ca and supplemental phytase in regard to total InsP hydrolysis is needed in order to find the optimum levels of added mineral P and Ca and phytase in a diet closer formulated to the requirement of available P.

Crop and proventriculus/gizzard

It is commonly accepted that formation of insoluble Ca-phytate complexes primarily occurs in the intestine. However, few studies indicated that Ca and phytate interactions also occur at acidic pH with the formation of soluble and insoluble Ca-phytate species (Selle et al., 2009). *In vitro*, increasing Ca concentrations reduced the liberation of P_i from sodium phytate by a microbial phytase at pH 2.5 and 6.5 (Tamim and Angel, 2003). In addition to the fact that a pH of 5 is pivotal to Ca-phytate precipitation (Selle et al., 2009) this indicates that Ca-phytate precipitation cannot be ruled out in the crop and proventriculus/gizzard. Ca can further elevate digesta pH because of limestone's very high acid binding capacity, which will favour Ca-phytate interactions and may further influence the activity of supplemented phytases depending on their pH activity spectrum (Selle et al., 2009). The pH in the crop increased from 4.89 to 5.32 when the Ca content in the diet was increased from 1.07% to 2.53% (Shafey et al., 1991). However, as already mentioned, in the present work the majority of phytate must have been highly soluble in the crop as shown by high $InsP_6$ hydrolysis (up to 80%) in the presence of added phytase (Manuscript 5). An increase in the molar ratio of Ca and $InsP_6$-P (by addition MCP and limestone) from 2.3:1 (Ca: 6.3 g/kg DM; $InsP_6$-P: 2.8 g/kg DM) to 2.7:1 (Ca: 7.6 g/kg DM; $InsP_6$-P: 2.8 g/kg DM) had no effect on $InsP_6$ hydrolysis in the crop irrespective of the presence or absence of supplemental phytase (Manuscript 5). Correspondingly, the accompanying inclusion of 0.8 g MCP-P per kg of diet did not affect $InsP_6$ hydrolysis in the crop of birds fed a maize-soybean meal based diet without or with added phytase (Manuscript 5). Similarly, supplementation of mineral P and Ca had no effect on concentrations of $InsP_6$ in the proventriculus/gizzard irrespective of phytase

supplementation (Annex 3). This confirms high solubility of phytate complexes in this acidic segment. Whether higher levels of mineral Ca and P than the included levels in the present work influence phytate hydrolysis in the crop or proventriculus/gizzard still needs to be investigated.

Small intestine

The pH in the small intestine of poultry was reported to range from 5.5 to 7.9 (Svihus, 2010). This is a favourable range for the formation of phytate complexes with Ca and other nutrients and precipitation. Precipitation is promoted by an increase in the dietary molar Ca:$InsP_6$ ratio which was further reported to increase the pH of ileal digesta (Shafey et al., 1991). In accordance, only negligible concentrations of water soluble $InsP_6$ were found in the small intestine of broilers (Leslie et al., 2006). A Ca solubility of only 11% was observed in the duodenum and jejunum (pH in digesta: 6.26) of broilers fed diets containing 9.0 g/kg Ca and 9.22 g/kg phytate (Pang and Applegate, 2007), likely because of Ca-phytate precipitation. Dietary Ca was also shown to reduce the mucosal phytase activity of chickens (McCuaig et al., 1972; Applegate et al., 2003). Moreover, Ca may influence the activity of specific types of phytases in the intestine because Ca was observed to affect the phytase activity of specific bacteria *in vitro* and Ca availability was reported to affect ileal bacterial populations in pigs (Shimizu, 1992; Metzler-Zebeli et al., 2010). In broilers it has been frequently demonstrated that increased dietary Ca concentrations (at constant tP levels) can reduce the $InsP_6$ hydrolysis with or without added phytase in the ileum or on the basis of excreta measurements (Mohammed et al., 1991; Tamim and Angel, 2003; Applegate et al., 2003; Tamim et al., 2004; Plumstead et al., 2008; Amerah et al., 2014). In accordance with this, the present work describes a reduction in $InsP_6$ hydrolysis and P net absorption measured in the duodenum/jejunum, lower ileum and excreta of broilers offered diets without or with 500 FTU/kg phytase when limestone and MCP/monosodium phosphate were added (Manuscript 2 and 3). Whether this depression in $InsP_6$ hydrolysis was due to the added mineral Ca or P, or both in combination, cannot be separated for this work and requires further studies. Studies should also investigate the effect of mineral Ca and P on the different sources of phytase in the digestive tract. The effect by mineral Ca could be counteracted by the addition of mineral chelators, such as citrate which was shown to increase the efficacy of phytase in increasing $InsP_6$ hydrolysis under simulated intestinal conditions of poultry (Zyla et al., 1995).

It is possible that mineral P reduced the activity of microbiota-associated, endogenous mucosal and supplemented phytase due to inhibition by the endproduct P_i. It may further have caused a down-regulation of the expression of phytase from microbiota-associated or endogenous mucosal phytase or a shift in the microbial population to fewer phytase-producing organisms. Such a shift may be related to the differences in P requirement and capability of different bacteria to produce phytase. Dietary mineral P was shown to reduce mucosal phytase activity (Abudabos, 2012a) and V_{max} of mucosal phytase (Onyango et al., 2006) in broilers and to change the intestinal microbial population in pigs (Metzler-Zebeli et al., 2010). Own results found reduced mucosal phytase activity close to the level of significance when MCP was supplemented in combination with 12,500 FTU/kg phytase (Huber et al., 2015). This pointed to reduced mucosal phytase activity due to high amounts of luminal P_i. *In vitro* studies showed a depression of phytase activity or synthesis by P_i in several bacteria (Shieh et al., 1969; Greiner et al., 1997). In broilers, mineral P combined with an increase in concentrations of dietary Ca has been frequently reported to reduce $InsP_6$ hydrolysis (Mohammed et al., 1991; Manangi and Coon, 2008). Manangi and Coon (2008) offered in another part of their study diets with a fixed Ca concentration to broilers and found a reduced $InsP_6$ hydrolysis based on excreta measurements with increasing levels of dietary non-phytate-P when diets contained no or 1,000 FTU/kg microbial phytase. Similarly, Leske and Coon (2002) reported decreased non-phytate-P retention in broilers fed diets without added phytase when the dietary tP concentration was increased from 0.4 to 1.1% at constant Ca level. These findings affirm that depressed $InsP_6$ hydrolysis in diets with or without 500 FTU/kg phytase in the present work besides Ca can also be related to a sole effect of P_i from the mineral P-source (Manuscript 2 and 3). Overall, the present findings and related literature emphasize that the frequently reported low phytate hydrolysis in broilers and differences in phytase efficacy between studies can to a large extent be related to the dietary mineral P and Ca concentrations used.

Manuscript 3 reported the effect of mineral P and Ca on $InsP_6$ hydrolysis and P net absorption to disappear at a level of 12,500 FTU/kg phytase. It was argued that the concentrations of liberated InsP-P_i and P_i from MCP were too low to trigger relevant product inhibition at such high concentrations of phytase. In addition, the level of $InsP_6$ hydrolysis was very high already in the crop (Manuscript 5) and proventriculus/gizzard where Ca was less likely to form precipitates and P_i had no influence on phytase efficacy. The inclusion of such high levels of phytase reduces the need for dietary mineral P. However, at present, a 25 fold increase of the current industry standard phytase level does not seem to be feasible. Since an

earlier study already indicated that lower levels of phytase (7,500 FTU/kg of an *Aspergillus* derived phytase) than 12,500 FTU/kg can cause an apparent $InsP_6$ hydrolysis beyond 90% (Nelson et al., 1971), further studies are needed to identify the level of phytase which maximizes $InsP_6$ hydrolysis and P accretion. Increasing the phytase dose from 500 to 12,500 FTU/kg resulted in enhanced P net absorption and performance (Manuscript 3). Additional supplementation of MCP to 12,500 FTU/kg phytase increased the amounts of net absorbed P and BW gain. These findings suggest additive effects of mineral P and phytase at such high doses of phytase. However, the present work did not involve urinary P excretion. The findings about these additive effects of mineral P and high doses of phytase are an important step in phytase research. This combination can ensure maximal InsP-P utilization, which reduces the need of dietary mineral P. At the same time, adequate amounts of mineral P and Ca can be added to cover the birds' P and Ca requirement without any negative effect on InsP-P utilization. However, this is just a first step towards optimization of phytase and mineral P input and P output from the economical and ecological perspective. Further studies are needed to find out the combined levels of phytase and specific mineral P and Ca sources which can maximize InsP degradation and increase the amount of absorbed and retained P and cover the bird's exact P requirement.

3.3.5 OTHER FACTORS

Various intrinsic and other extrinsic factors than mineral P and Ca can influence the activity or action of microbiota-associated, endogenous mucosal, intrinsic plant and supplemental phytases.

Factors affecting the activity of endogenous mucosal and microbiota-associated phytases

Intrinsic factors such as age, species and gender of poultry were shown to affect endogenous mucosal phytase activity (Maenz and Classen, 1998; Applegate et al., 2003; Abudabos, 2012a,b). Genetics and breed may also influence phytase activity, as higher endogenous mucosal phytase activity was found in fast- compared to slow-growing broilers (Abudabos et al., 2012a). Zhang et al. (2003) reported a heritability of 0.1 for phytate-P availability in broilers. Correspondingly, Beck et al. (2014) found a heritability of 0.09 for P utilization in quails. This could be in part related to genetic differences affecting the activity of endogenous mucosal and microbiota-associated phytases. Such differences were also indicated by large individual variation in phytate-P utilization within a broiler strain under similar environmental conditions (Punna and Roland Sr., 1999). Differences in P-utilization between different broiler strains were also described (Edwards Jr., 1983). Breeding and selection for high

phytate-P utilization may increase phytase activity in the intestine. The regulation mechanisms for activity of endogenous mucosal and microbiota-associated phytases by different influencing factors are still largely unknown, but quite different mechanisms can be assumed. Factors which affect phytase activity could influence gene transcription or protein translation and thereby the amount of enzyme present. The activity of different isoenzymes could be influenced which results in changes of kinetic parameters. Factors could further have an effect on protein folding or trigger covalent modifications of phytases. Phytase gene activity could in part be regulated epigenetically. Identification of factors, especially nutrient factors, which alter epigenetic regulation of phytases in the digestive tract, could be a valuable tool to increase phytase activity in the intestine. Respective nutrient constraints in early development or further breeding of animals with epigenetically modified intestinal phytase activity could ensure high activities of endogenous mucosal and/or microbiota-associated phytases. In this context, apart from dietary Ca and P_i, an influence of dietary Mg, Fe, and vitamin D on mucosal phytase activity was reported in broilers (Davies et al., 1970; McCuaig et al., 1972; Maenz and Classen, 1998; Abudabos et al., 2000; Applegate et al., 2003). Phytate-induced phytase production was reported for several bacteria and increased mucosal phytase activity was shown with dietary phytate in rats (Yang et al., 1991; Greiner et al., 1997; Kerovuo et al., 1998; Lopez et al., 2000; Lan et al., 2002). Intestinal mucosal phytase in broilers was reduced with phytase supplementation (Abudabos, 2012a) which suggests an impact of less accessible phytate in relation with higher P availability on the activity of endogenous mucosal phytases. As aforementioned, own results pointed to reduced mucosal phytase activity with supplementation of MCP in combination with 12,500 FTU/kg phytase (Huber et al., 2015). It was supposed that protein expression was down-regulated in presence of more P_i. The composition of microbiota in the ileum of broilers also changed with phytase supplementation (Camarinha-Silva et al., 2015; Witzig et al., 2015). In addition, it was shown that the microbiota from the environment of high-phytate content (vegetarians' intestine) was the most effective in hydrolyzing phytate in human (Markiewicz et al., 2013). Thus, high dietary phytate concentrations seem to increase the potential of intestinal microbiota to hydrolyse phytate. This suggests that microbiota adapt to dietary phytate and available P concentrations by a changing proportion of phytase- and non-phytase producing bacteria and probably adapting phytase production.

Factors affecting the efficacy of phytases of different origin

Apart from endogenous mucosal phytase and microbiota-associated phytases added microbial and intrinsic plant phytases can be affected in their activity by different factors. The activity of purified microbial phytase, intrinsic plant phytases and endogenous mucosal phytase from BBV of broilers were reported to be affected by different cations *in vitro* (Gibson and Ullah, 1988; Greiner et al., 1997, 1998; Maenz and Classen, 1998; Nakano et al., 1999; Konietzny and Greiner, 2002; Abudabos, 2012a). Besides such cations in the diet, other dietary factors may restrict the action and activity of phytases of different origin in the digestive tract. Several dietary factors influencing viscosity and retention time of digesta can affect the contact between phytase and phytate in the gut. In this context, increased $InsP_6$ hydrolysis by slower passage rate due to shorter day length was shown in the ileum and excreta of broilers fed diets with added phytase (Leslie et al., 2006). Especially in the crop, retention time and the linked moistening of the digesta, which is essential for enzyme mobility, solubility of phytate and enzyme, or both (Svihus, 2010), determine the efficacy of dietary phytases. *In vitro* studies have shown that the level of $InsP_6$ hydrolysis by intrinsic plant phytase in part depends on incubation time (Carlson and Poulsen, 2003; Blaabjerg et al., 2010a). $InsP_6$ hydrolysis was low at 25% and 35%, but dramatically increased at 45% moisture up to 86% during incubation of a wheat-based diet with supplemental phytase (Denstadli et al., 2006). Within a retention time of 60 min in the crop of broilers fed wheat-based diets supplemented with an *E. coli* derived phytase an $InsP_6$ hydrolysis of approximately 45% and a moisture content of approximately 50% were observed; $InsP_6$ hydrolysis further increased with prolonged retention time and linked increasing moistening of digesta (Svihus et al., 2010).

Factors such as particle size or feed processing may influence phytase activity and phytate accessibility or solubility. Especially in the crop, feed particle size may be a decisive factor for the accessibility of phytate for phytase. Grinding of wheat-based feed samples without added phytase was shown to increase the amount of dialyzable phosphate under simulated conditions of broilers' intestine (Zyla et al., 1999). Authors concluded that the mechanical breakdown of plant tissue increased the access of enzymes to wheat phytate. Phytase activity in wheat is located in the aleurone layer (40%), endosperm (34%), scutellum (15%), germ (3%), epidermal layers (2%) and testa and cross layers (5%) (Peers, 1953). Phytate in wheat is predominantly located in the aleurone layer (87%); the majority of the remaining phytate is present in the germ (O'Dell et al., 1972). In the aleurone layer phytate and phytases are enclosed by the thick cell walls and other surrounding structures. Hence, when those and

other structures in the grain are intact, hydrolysis of phytate is probably limited to the proportion of phytase localised in the same grain fraction. Blaabjerg et al. (2010a) demonstrated that phytases from ground wheat degraded $InsP_6$ from ground soybean meal (both set to 3 mm between the rolls in the mill) and enzymes and substrate are able to get in contact. This suggests that either one of them or both were released from the storage site (Blaabjerg et al., 2010a). It can be concluded that grinding of wheat can promote the contact between phytases and phytate from different wheat fractions and between wheat phytase and phytate from soybean meal. Similarly, grinding of wheat in the present work (ground through a 2-mm sieve) (Manuscript 5) probably facilitated the contact of phytate and phytase which promoted high hydrolysis of dietary $InsP_6$ by intrinsic wheat phytase in the crop. Correspondingly, changing the screen size from 3 to 1 mm increased the relative phytate hydrolysis rate with microbial phytase in maize by 22%/h during incubation in water at 38°C (Ton Nu et al., 2014). In accordance, phytase supplementation increased ileal P digestibility and toe ash content in broilers fed a medium particle size maize-based diet, but had no effect in those fed a coarse particle size maize-based diet (Amerah and Ravindran, 2009). In contrast, Kasim and Edwards Jr. (2000) observed that increased particle size and supplemented phytase improved P and Ca retention in broilers without any interaction and concluded that this was due to prolonged retention time in the gut. However, in the present experiments, particle size of the diet, especially of the included maize meal, was relatively small. This might have contributed to a high accessibility and hydrolysis of phytate in the crop due to enhanced contact between phytate and supplemental phytase and exposure of the possibly inaccessible fractions (Manuscript 1 and 5). Particle size was probably further reduced during the pelleting process as described by Svihus et al. (2004) and Amerah et al. (2007). Accessibility of phytate from soybean meal might have been further increased as a consequence of dehulling, oil removal and processing during solvent extraction. In this context, solvent extraction of rapeseed was reported to cause fracture of cell walls leaving behind an amorphous protein matrix still embedded with phytin globoids (Yiu et al., 1983). Correspondingly, microbial phytase had a great effect on phytate degradation in solvent-extracted soybean meal but no effect in wheat (Blaabjerg et al., 2010a). The authors suggested that this was in part related to structural changes caused by solvent extraction which influenced the possibility for contact between phytate and the added phytase.

Various *in vitro* studies already indicated that the efficacy of phytase is also dependent on the feedstuffs used. Microbial phytase had no influence on $InsP_6$ hydrolysis in wheat, but increased $InsP_6$ hydrolysis in rapeseed cake and to a higher extent in soybean meal

(incubation in water at 20°C) (Blaabjerg et al., 2010a). The P_i release in the presence of microbial phytase was greatest for soybean meal, intermediate for a maize-soybean meal-based diet and least for maize during incubation at pH 4.5 and 40°C (Adeola et al., 2004). In contrast, a recent study reported that the effect of microbial phytase on the relative phytate degradation was higher in maize than in soybean meal during incubation in water at 38°C (Ton Nu et al., 2014). Since the initial phytate content was higher in soybean meal than in maize the amount of phytate degraded per hour was lower in maize than in soybean meal (Ton Nu et al., 2014) which corresponds to the results of Adeola et al. (2004). Variations in phytase efficacy between different feedstuffs may be related to the concentration, structure and storage site of phytate in a particular ingredient (Selle and Ravindran, 2007). These factors may determine accessibility of phytate and interactions with other nutrients, and thus, phytate solubility. Differences in the content and localization of phytate between different feedstuffs were already described in detail in Chapter 1. De Boland et al. (1975) detected differences in solubility of phytate between different feedstuffs. In accordance with the described *in vitro* studies, differences in $InsP_6$ hydrolysis were demonstrated in the excreta of broilers fed diets containing soybean meal, corn, wheat, wheat middlings, barley, defatted rice bran or canola meal with or without added phytase (Leske and Coon, 1999). In the present work, a higher increase in $InsP_6$ hydrolysis by supplemented phytase was found in the crop of birds fed a maize- compared to those fed a wheat-based diet (Manuscript 5). These differences in efficacy of supplemented phytase in the crop were suggested to be primarily related with higher accessibility of phytate for added phytase in maize compared to wheat due to different storage sites (germ vs. aleurone layer) (Manuscript 5). This affirms the results of Blaabjerg et al. (2010a, 2012) which showed no effect of phytase addition on phytate degradation in soaked wheat or barley (heat-treated or not). In accordance to this, added phytase had a high effect on $InsP_6$ hydrolysis in soybean meal, an intermediate effect in a wheat-soybean meal diet and no detectable effect in wheat (Blaabjerg et al., 2007). From these results it can be concluded that added phytase in the crop of birds fed a wheat-based diet primarily acts on phytate from soybean meal since the contact to phytate from wheat seems to be restricted. However, in the present work differences in efficacy of supplemented phytase between the diets seem to disappear in the ileum (Manuscript 3 and 4).

Overall, it was shown that many factors other than dietary mineral P and Ca can influence the efficacy of phytases of different origin in the digestive tract. Differences in these factors can also explain the variations in $InsP_6$ hydrolysis in broilers and differences in the efficacy of dietary phytases observed in different experiments. Identification of those factors, their

interactions and their relevance requires further experimental work. Better knowledge about the substrate for phytase is needed. Binding forms, solubility and localization of phytate and its accessibility for phytases of different origin in different raw materials and mixed diets should be investigated. First information about the efficacy of different phytases in different diets could be obtained from *in vitro* studies.

3.4 RELEVANCE OF LOWER INOSITOL PHOSPHATES

Up to now, only few studies investigated the appearance of lower InsPs with different degree of phosphorylation in the digesta or excreta of poultry. To the best of the author's knowledge, the present work is the first which determined positional InsPs in the digesta of poultry. The relevance of lower InsPs and *myo*-inositol in the digesta of poultry has been rarely discussed in the literature. Therefore, the present chapter discusses the relevance of lower InsPs and *myo*-inositol in the digestive tract of poultry.

Provision of available P

Alongside hydrolysis of $InsP_6$ the degradation of lower InsPs is relevant for the supply of available P. In the present work, the maximal proportion of $InsP_{3\text{-}5}$-P in tP was found in lower ileum (12% $InsP_{3\text{-}5}$-P in tP) in the presence of MCP and 500 FTU/kg of an *E. coli* derived phytase (Manuscript 3). P net absorption was 50% for this diet. In this example, a complete liberation of $InsP_{3\text{-}5}$-P could result in an increase in P net absorption of 6% if all released P_i is absorbed. In the absence of MCP and supplemented phytase, an average proportion of 5% $InsP_{3\text{-}5}$-P in tP and P net absorption of 52% was found in the lower ileum (Manuscript 3). For these values, complete liberation of $InsP_{3\text{-}5}$-P could result in an increase in P net absorption of only 2%. This difference demonstrates that a reduction in the degradation of lower InsPs can reduce the provision of available P. In the present study, it was suggested that phosphatases further degraded lower InsPs formed by supplemented phytase despite of the presence of MCP. The activity of different phosphatases in the intestine depends on different dietary and animal factors and may be much lower resulting in even much higher concentrations and proportions of lower InsPs in tP in other studies compared to the present work. It can be concluded that the degradation of lower InsPs which is affected by different factors can significantly contribute to the supply of available P.

Other roles of lower InsPs

The relevance of the degradation and presence of lower InsPs in the digestive tract may extend beyond the supply of available P. The occurrence of specific lower InsPs and *myo*-inositol is relevant for cellular processes, nutrient absorption and physiological or immunological functions. In addition to increased utilization of InsP-bound nutrients this can explain the so called "extraphosphoric effect" of supplemented phytase. The presence of specific InsPs or *myo*-inositol in the intestinal lumen may be directly relevant for P_i and Ca transport processes or for receptor-mediated intracellular processes affecting nutrient absorption. Huber et al. (2015) were the first who detected significant correlations between concentrations of specific InsPs in the intestinal digesta and the protein expression of intestinal phosphate transporters in broilers. This study was an accompanying study to the one described in Manuscript 3 where MCP and phytase supplementation generated different InsP pattern in the small intestine. Hence, besides regulation by available P, these results indicate a potential modulation of transporter expression by lower InsPs. In this context, endogenous mucosal phosphatases may be important. Despite of their unknown contribution to luminal hydrolysis of InsPs, endogenous mucosal phosphatases may be of local relevance increasing the amount of absorbable P, *myo*-inositol and specific InsPs near the surface of the mucosa.

$InsP_6$ and its derivatives are ubiquitous in eucaryontic cells. It has already been shown that the number and position of phosphate groups of InsPs are relevant for physiological functions such as transmembrane signalling, intracellular Ca mobilization (De Lisle et al., 1995), and extracellular Ca influx (Neher, 1992). In particular, Ca release from intracellular stores is triggered by the second messenger $InsP_3$, and regulation of this process is critically important for cellular homeostasis and major cellular functions. InsPs are also known to contribute to immune functions in the intestinal epithelium via the regulation of pro-inflammatory cytokine secretion and the enhancement of natural killer cell activity (Urbano et al., 2000; Wawszczyk et al., 2012). Specific InsPs were shown to be involved in haemoglobin modulation in erythrocytes (Irvine and Schell, 2001). Intracellularly, InsPs are involved in gene regulation, mRNA export and DNA repair (York et al., 1999; York, 2006). In addition, a supporting function in the process of cell division and differentiation was described for InsPs (Berridge and Irvine, 1989). Correspondingly, InsPs formed by different dietary phytases in the digestive tract of broilers were assumed to possess physiological relevance since supplementation of those phytases resulted in different physiological responses (Zyła et al., 2004). However, whether InsPs in the digesta influence intracellular InsPs or trigger other

intracellular processes which provoke a physiological response is still unknown. Since *de novo* synthesis of InsPs was described within mammalian cells (Irvine and Schell, 2001; Letcher et al., 2008) InsPs may be formed intracellularly on demand.

Myo-inositol is an essential factor for growth and homeostasis of some microorganisms and human cells (Liu et al., 1998). It is known as an osmolyte in the central nervous system and a lipotropic factor. It represents the precursor of a second messenger in the cell signalling system (Majerus, 1992) and may be relevant for various metabolic processes. *Myo*-inositol was reported to show an insulin mimic effect, since it stimulates the translocation of the most important insulin-sensitive glucose transporter to the plasma membrane and therefore represents a potential therapeutic in the prevention and treatment of diabetics (Dang et al., 2010; Yamashita et al., 2013). The potential of dietary *myo*-inositol to improve the performance of broilers was already shown (Zyla et al., 2013; Cowieson et al., 2013). Supplementation of high doses of a phytase (1000-3000 U/kg) can substantially increase plasma inositol concentrations in broilers (Cowieson et al., 2014). Authors suggested that increased plasma inositol concentrations may be beneficial in nutrient transport and protein deposition. Similarly, *myo*-inositol concentrations in the gizzard were increased by high doses of supplemental phytase and correlated positively with body weight gain and negatively with feed conversion ratio (Walk et al., 2014). Authors concluded that the benefits of high doses of phytase in body weight gain and feed efficiency were in part related to provision of *myo*-inositol.

Whether specific lower InsPs in the digesta can be absorbed or internalised by the epithelium of the digestive tract and further by the epithelium of other organs or by other tissues and directly provide P and contribute to cellular and metabolic processes is still unclear. They may also activate receptors or may be metabolized by phosphatases and rephosphorylated by kinases intracellularly in the mucosa, blood and other tissues. In few studies radiolabelled phytate (labelled at the inositol ring) was administered to rats and radioactivity was recovered in blood, organs, urine, bones and gut (Nahapetian and Young, 1980; Sakamoto et al., 1993). This suggests that $InsP_6$, lower InsPs or *myo*-inositol was absorbed, eventually metabolised and distributed to various tissues. More recent studies showed an uptake of $InsP_6$ in HeLa and MCF-7 breast cancer cells and its partially intracellular degradation to lower InsPs (Ferry et al., 2002; Vucenik and Shamsuddin, 2006). Further, a dependence between the orally administered phytate and phytate concentrations in the urine and plasma was reported in humans and rats (Grases et al., 2000a,b, 2001a,b,c, 2002). In contrast, Letcher et al. (2008)

detected no $InsP_6$ in platelet-free plasma, serum and urine. To conclude, the majority of published data indicate that phytate may be internalized in cell culture systems and absorbed by the intestinal epithelia in rats and humans. However, whether absorption occurs in the intestine of avian species still needs to be clarified. The question about cellular uptake of lower InsPs requires further experimental work. The effect of different InsPs and *myo*-inositol on cellular and transport processes, on metabolic processes in blood, different tissues and organs also should be further investigated. This, in addition to the influence of dietary factors on the occurrence of these derivatives in the digesta can open a fruitful research area for the future. In this context, it was already suggested for humans that a controlled degradation of $InsP_6$ forming desired specific InsPs with respective benefits is a future challenge (Sandberg and Andlid, 2002). Perhaps, in poultry production high $InsP_6$ hydrolysis in combination with controlled production of specific beneficial lower InsPs will be a compromise.

3.5 CONSEQUENCES FOR P EVALUATION IN POULTRY

The findings of this thesis support the need for current P evaluation systems to be revised. The affirmation that $InsP_6$-P can be highly available for broilers even without detectable dietary phytase activity (Manuscript 1-3) demonstrates that the simple differentiation in non-phytate P and phytate P together with the assumption that only non-phytate P is available for poultry (NRC, 1994, GfE, 1999) is outdated. The supply of available P, as defined by WPSA (2013), linked with phytate degradation until the end of the small intestine should be taken into account in diet formulation to cover P requirements and simultaneously avoid excessive use of mineral P and P excretion.

However, the magnitude of phytate degradation in broilers was shown to depend on different dietary factors. Technological treatment or processing of feed and feed ingredients may affect P availability. In this context, microwave treatment of wheat meal increased phytate hydrolysis but had no effect on P net absorption in the lower ileum of broilers (Manuscript 4). Different structures between raw materials may similarly affect phytate accessibility and thus degradation. Diets differing in intrinsic phytase activity showed no differences in $InsP_6$ hydrolysis in the lower ileum (Manuscript 2 and 4), but much higher intrinsic phytase activities than in the present work are supposed to increase $InsP_6$ hydrolysis. Varying P_i and $InsP_6$-P concentrations between raw materials could also alter phytate degradation. As recommended by the standard protocol of the WPSA (2013), the precise supply of dietary P to meet the specific needs of an animal requires knowledge about the availability of P from

different feed raw materials. Feed tables describing available P based on prececal digestible P from different raw materials and different cereal varieties would be a first improvement compared to the current practice. Differences in P availability from different raw materials due to processing or particle sizes should also be investigated.

Because dietary ingredients, such as mineral P and Ca (Manuscript 2 and 3) can alter $InsP_6$ hydrolysis, the amount of available P provided from different ingredients cannot simply be summed up in a mix of these ingredients. There are various interactions between different ingredients in regard to $InsP_6$ hydrolysis and the amount of released P which are currently not yet understood. Thus, better knowledge about the separate effects of different factors and their interactions is needed to predict P availability from different mixed diets in future. In regard to phytase supplements, the World's Poultry Science Association already reported that they need to be evaluated as to what extent they are able to increase the availability of P from certain raw materials and mixed diets (WPSA, 2013). Results of the present thesis confirm that supplemental phytases of different origin may differ in efficacy as shown by differences in P net absorption measured in the lower ileum (Manuscript 1). Dependence of phytase efficacy on raw materials was indicated by a different increase in $InsP_6$ hydrolysis by supplemental phytase in the crop in a maize- compared to a wheat-based diet (Manuscript 5), but this difference disappeared in the lower ileum (Manuscript 3 and 4). Since phytases of different origin may show synergistic effects on InsP degradation (Manuscript 5) or compete for the substrate, combination of different phytases will not result in linear additive responses in regard to P release. It was further demonstrated that the efficacy of supplemented phytase in phytate degradation and P net absorption measured in the small intestine can be reduced by dietary mineral P and Ca (Manuscript 2 and 3). The effect of added mineral P and Ca on phytase efficacy depends on the dose of supplemented phytase (Manuscript 3). In contrast to standard phytase levels, a very high dose of phytase and mineral P may in part exert additive effects on the amount of available P in the intestine (Manuscript 3). However, these interactions not only show the dependence of phytase efficacy on mineral P and Ca. Vice versa, these interactions demonstrate that values for the use of mineral P must be adjusted for the decline in total InsP hydrolysis they cause in the respective basal diet (Manuscript 2). But this decline and the use of mineral P may differ in the presence of different levels of supplemented phytase (Manuscript 3). This indicates that availability of non-phytate P depends on dietary ingredients, which contradicts the conception of the current German P evaluation systems that availability of non-phytate is constant by 70% (GfE, 1999).

Overall, it can be clearly deduced for P evaluation systems that additivity of different P providing ingredients in mixed diets is not given. Interactions between different dietary influencing factors must be considered in future predictions of available P from specific diets. Especially the separately determined amount of prececal digestible P provided by specific raw materials, a mineral P source and by specific phytases cannot simply be added for a diet containing these ingredients. However, further experimental work is needed evaluating such interactions between different types and levels of phytases and levels of different mineral P sources supplemented to specific basal diets.

3.6 PERSPECTIVES FOR FUTURE RESEARCH

In the animal studies of the present work, the $InsP_6$ hydrolysis and appearance of lower InsPs resulted from combined effects of dietary, microbiota-associated and endogenous mucosal phosphatases. The contribution of each of these players is still unknown to date and has been controversially discussed. Therefore, better knowledge of the InsP degradation by each of them separately from the other is needed. This would also be a next step to understand better the interactions between the different phytase sources in regard to InsP degradation. This knowledge can represent a valuable tool to find in a later step solutions to maximize InsP degradation in broilers.

The use of an *in vitro* system which mimics conditions in different segments of the digestive tract, such as pH, temperature, presence of proteases and retention time of digesta and the consecutive passage of the different segments could help to differentiate between InsP degradation by different sources of phytase. The major advantage of such an approach is to characterize the degradation of phytate within the feed matrix by supplemented microbial and intrinsic plant phosphatases under simulated conditions of the digestive tract independent of microbiota-associated and endogenous mucosal phosphatases. Better understanding of the influence of other dietary factors and their interactions can also be gained using such an *in vitro* system. For example it could help to clarify whether MCP affected the activity of supplemented phytase besides microbiota-associated and mucosal phosphatases. In addition, it can be an approach to screen and predict the action and characteristics of different types of phytases in different segments of the digestive tract. This knowledge can be helpful for further development of microbial phytase preparations. The efficacy of phytases can be primarily predicted for the anterior segments of the digestive tract under simulated conditions, where

InsP degradation is mainly related to dietary phytases. This prediction may help to find the optimal dosage of specific phytases within different diets with varying ingredients.

The contribution of microbiota-associated and endogenous mucosal phosphatases to InsP degradation in the digestive tract may be separated in animal trials using germfree and non-germfree chickens (Kerr et al., 2000; Sandberg and Andlid, 2002). Such trials can give information about the InsP degradation by endogenous mucosal phosphatases alone in the germfree animals compared with InsP degradation by the combination of endogenous mucosal and microbiota-associated phosphatases in the non-germfree animals. In addition, the effect of different factors on InsP degradation by mucosal phosphatases alone or by the combination of endogenous mucosal and microbiota-associated phosphatases can be tested. To study the InsP degradation by microbiota-associated phosphatases alone in non-germfree animals, knockout animals may be developed in which genes encoding for mucosal phosphatases (or in a first step encoding for mucosal phytases) were inactivated could be used. However, a prerequisite is the identification of the respective gene sequences. Further, the use of factors (perhaps of dietary origin) suppressing expression of the genes encoding for mucosal phosphatases or suppressing protein translocation of mucosal phosphatases in non-germfree animals is conceivable. However, this requires the identification of factors or the dose of such factors which results in complete suppression of the gene expression or protein translocation.

Another tool to maximize InsP degradation may be animal breeding and genetics (Diarra et al., 2010). Selection for birds with the trait of high phytate hydrolysis and P utilization and further breeding of these animals can be one direction to increase phytate hydrolysis and P utilization in broilers (Zhang et al., 2003). From trials with individual animals in which as many data as possible are collected could be deduced which parameters are linked with high phytate hydrolysis and P utilization (Beck et al., 2014) (e.g. mucosal phytase activity, the microbial composition/microbiome or P transporters). An accompanying genotyping can help to identify the gene loci which are relevant to the respective trait and parameters. Later, respective modification of gene activity/expression by genetic engineering or epigenetic alterations could be envisaged. In this context, nutrigenetical changes in the early stage development (prenatal or postnatal) could be of interest.

Plant breeding and genetics similarly represent an important area in future research towards maximization of InsP degradation in broilers (Diarra et al., 2010). One direction can be selection and breeding for high intrinsic plant phytase activity (Diarra et al., 2010).

Modification of the activity/expression of genes encoding intrinsic plant phytase by genetic engineering which may realize high intrinsic plant phytase activity is also conceivable (Brinch-Pederson et al., 2002). The introduction of genes encoding specific phytases in cereals may be another strategy to maximize InsP degradation without supplemental phytase in the digestive tract (Pen et al., 1993; Zhang et al., 2000).

Furthermore, future research should clarify more precisely whether specific InsPs can be absorbed or internalised by the epithelium of different organs and by other tissues. First information could be gained from studies using cell lines, Ussing chamber systems or preparations of apical membrane vesicles. Moreover, it is not known, which and to what extent InsPs in the digesta may be absorbed and potentially exert a physiological response (Sandberg and Andlid, 2012). Hence, the effect of different InsPs and *myo*-inositol in the digesta on cellular and transport processes, on InsPs and *myo*-inositol in the blood, and on metabolic processes in blood, different tissues and organs of broilers should be investigated. To get the linkage between the influence of dietary factors on the occurrence of these InsPs in the digesta and those processes the cooperation between animal nutritionists, cell biologists and physiologists is needed.

3.7 CONCLUSIONS

- Broilers and their gut microbiota have a strong potential to hydrolyse $InsP_6$ in the small intestine in low-P and low-Ca diets. Since endogenous mucosal and microbiota-associated phytases have a high capacity to hydrolyse $InsP_6$ they are supposed to be the major contributors to phytate hydrolysis in the small intestine of broilers. But the differentiation between these two origins of phytases, and between phytases from different microorganisms and their contribution to phytate hydrolysis needs further investigation.
- High $InsP_6$ hydrolysis by intrinsic plant phytase in the crop can significantly contribute to the total $InsP_6$ hydrolysis. But in broilers which have a high capacity to hydrolyse phytate in the gut, intrinsic plant phytase cannot increase total phytate hydrolysis. The use of cereal cultivars with high intrinsic phytase activity or genetically engineered cereals for increased phytase activity could result in high ileal values, exceeding $InsP_6$ hydrolysis by mucosal or microbiota-associated phytases.

- Applying doses of supplemental phytase that are several fold higher than the industry standard can increase $InsP_6$ hydrolysis in the small intestine up to more than 90%. The majority of hydrolysis occurs in the anterior segments. Such high hydrolysis in the crop and proventriculus/gizzard promotes high $InsP_6$ hydrolysis in the digestive tract of birds fed mineral Ca and P because mineral Ca and P seem to reduce $InsP_6$ hydrolysis primarily in the small intestine. However, the presence of $InsP_6$ and lower InsPs may be relevant for varying metabolic processes. Further studies should investigate the relationship between different InsPs in the digesta and such processes.
- Different factors can influence phytate hydrolysis, the appearance of lower InsPs and the efficacy of dietary phytases in the digestive tract. Better knowledge about the influence of these factors and their interactions in regard to the activity and action of phytases of different origin in the lumen of the digestive tract is needed. Coordination and adjustment of these influencing factors in order to optimize efficacy of phytases will be a major challenge in future research. Future research must address the "big picture". In accordance with the interdisciplinary emphasis of bioeconomy the cooperation of animal nutritionists, microbiologists, physiologists, animal and plant breeders, and biotechnologists is needed to find solutions.
- P evaluation systems have to be reconsidered. The supply of available P linked with phytate degradation until the end of the small intestine has to be taken into account in diet formulation. Since additivity of different P providing ingredients in mixed diets is not given, interactions between different dietary influencing factors must be considered in future predictions of available P from specific diets.

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CHAPTER 4

INCLUDED MANUSCRIPTS

MANUSCRIPT 1:

Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplemented phytases in different segments of the digestive tract of broilers

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RESEARCH ARTICLE

Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplemented phytases in different segments of the digestive tract of broilers

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Abstract

The objective was to characterise degradation of *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$) and formation of inositol phosphate (InsP) isomers in different segments of the broiler digestive tract. Influence of an *Aspergillus niger* (PhyA) and two *Escherichia coli*-derived (PhyE1 and PhyE2) phytases was also investigated. A total of 600 16-d-old broilers were allocated to forty floor pens (ten pens per treatment). Low-P (5·2 g/kg DM) maize–soyabean meal-based diets were fed without (basal diet; BD) or with a phytase added. On day 25, digesta from different digestive tract segments were pooled per segment on a pen-basis, freeze-dried and analysed for P, InsP isomers and the marker TiO_2. $InsP_6$ degradation until the lower ileum (74 %) in BD-fed birds showed a high potential of broilers and their gut microbiota to hydrolyse $InsP_6$ in low-P diets. Different InsP patterns in different gut segments suggested the involvement of phosphatases of different origin. Supplemented phytases increased $InsP_6$ hydrolysis in the crop ($P < 0{\cdot}01$) but not in the lower ileum. Measurements in the crop and proventriculus/gizzard confirmed published *in vitro* degradation pathways of 3- and 6-phytases for the first time. In the intestinal segments, specifically formed $InsP_{4–5}$ isomers of supplemented phytases were still present, indicating further activity of these enzymes. *Myo*-inositol tetrakisphosphate ($InsP_4$) accumulation differed between PhyE1 and PhyE2 compared with PhyA in the anterior segments of the gut ($P < 0{\cdot}01$). Thus, the hydrolytic cleavage of the first phosphate group is not the only limiting step in phytate degradation in broilers.

Key words: **Inositol phosphate isomers: Phytate hydrolysis: Phytases: Broilers**

Phytate represents the primary storage form of P in plant seeds. It is defined as any salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) or $InsP_6$). The utilisation of $InsP_6$-P depends on $InsP_6$ hydrolysis because P absorption occurs mainly as orthophosphate[1]. $InsP_6$-hydrolysing enzymes such as phytases (*myo*-inositol hexakisphosphate phosphohydrolases) catalyse the hydrolytic cleavage of $InsP_6$ and its salts via several phosphorylated intermediary products (*myo*-inositol pentakis-, tetrakis-, tris-, bis- and monophosphate) down to *myo*-inositol. The International Union of Pure and Applied Chemistry/International Union of Biochemistry differentiates among three types of phytases: 3-phytases (*EC* 3.1.3.8), 4-/6-phytases (*EC* 3.1.3.26) and 5-phytases (*EC* 3.1.3.72), a classification that refers to the initiating position on the inositol ring during *in vitro* $InsP_6$ dephosphorylation. The 6-phytases usually originate from plants and initiate hydrolysis at the D-4 (L-6) position of $InsP_6$[2]; 3-phytases are usually of microbial origin (starting hydrolysis at the D-3 (L-1) position), such as the fungal *Aspergillus niger* phytase[3–5] or the bacterial *Pseudomonas* phytase[2]. However, *Escherichia coli* phytase as an exception was characterised as a 6-phytase (starting hydrolysis at the D-6

Abbreviations: BD, basal diet; InsP, inositol phosphate; $InsP_3$, *myo*-inositol trisphosphate; $InsP_4$, *myo*-inositol tetrakisphosphate; $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); PhyA, *Aspergillus*-derived phytase Finase® P; PhyE1, *Escherichia coli*-derived phytase Quantum®; PhyE2, *E. coli*-derived phytase Quantum® Blue; tP, total P.

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(L-4) position)[6,7]. We are not aware of any study that has investigated whether this *in vitro*-based classification is reflected also in the pathways of $InsP_6$ degradation in the more complex and variable environment of the digestive tract of broilers.

$InsP_6$-P has long been assumed to be poorly used by avian species because of the lack of sufficient endogenous $InsP_6$-hydrolysing enzymes and the denaturation of intrinsic plant phytases in the stomach and during feed manufacture. In broilers, although the activity of endogenous mucosal phytase in the small intestine has been described[8–10], its contribution to $InsP_6$ hydrolysis has been considered almost negligible. Although some more recent studies have indicated that $InsP_6$-P is highly available for broilers[11], the origin of phytase activity in the digestive tract is controversial. Some authors have suggested that endogenous mucosal phytase of the small intestine is very capable of high $InsP_6$ hydrolysis[12,13]. Others hypothesise that $InsP_6$ is hydrolysed by phytases produced by micro-organisms present in the small intestine and particularly in the caeca[14,15].

It is not known which positional inositol phosphate (InsP) isomers are formed by different phytases in the digestive tract of avian species. Some studies have described different positional isomers of $InsP_6$ degradation products in several segments of the digestive tract in pigs[16–18], but the variations in digestive tract physiology and anatomy between pigs and birds (for example, passage rate, digesta viscosity, presence of a crop, variable pH values) caution against assuming that findings in pigs will be similar to that in broilers. One consequence of the uncertainties regarding the availability of $InsP_6$-P is that commercial poultry diets are supplemented with mineral P sources. This costly supplementation increases the P concentration in excreta, which may contribute to environmental problems such as eutrophication of surface waters and exhaustion of global raw phosphate resources[19]. An understanding of the rate of degradation of $InsP_6$ to the different positional InsP isomers along the digestive tract would enable a more precise alignment of the feed composition to the birds' P requirements and thus increase the likelihood of averting a 'potential planet phosphate crisis'[20]. Phytases of different origin, varying in their properties, such as pH optimum, proteolytic stability and kinetic efficiency, may differ in effectiveness with transit as the conditions along the digestive tract change. The *in vitro*-determined pH optimum of most phytase supplements is particularly aligned to the conditions of the anterior segments of the digestive tract[21].

The first objective of the present study therefore was to characterise $InsP_6$ hydrolysis and formation of InsP isomers in different segments of the digestive tract of broilers. The second objective was to investigate the $InsP_6$ degradation pattern of different phytase additives and their effectiveness in releasing phosphate in broilers and to compare the findings with known *in vitro* properties. In the absence of supplementary phytase, $InsP_6$ was hypothesised to be mainly hydrolysed in the posterior intestinal segments of the digestive tract. In contrast, the different phytase supplements were expected to result in greater overall rates of $InsP_6$ hydrolysis and to elicit different InsP patterns in the anterior segments.

Materials and methods

Experimental diets

The basal diet (BD) was calculated to contain adequate levels of all nutrients according to the recommendations of the Gesellschaft für Ernährungsphysiologie (Society for Nutrition Physiology)[22] with the exception of Ca and P. It was mainly based on maize and solvent-extracted soyabean meal (Table 1). Ingredients were chosen to obtain low concentrations of total P (tP), high proportions of $InsP_6$-P in tP and low intrinsic phytase activity. Concentrations of Ca and tP were calculated to be 7·9 and 5·0 g/kg of DM, respectively, and these levels were confirmed by analyses (Table 1). Titanium dioxide (TiO_2) was included at a rate of 5 g/kg as the indigestible marker, and the intended Ti concentration was confirmed by analysis.

Diets were prepared in the certified feed mill facilities of Hohenheim University's Agricultural Experiment Station. The BD was mixed in one lot and divided into four equal parts. One part remained without phytase supplementation (BD). The other parts were supplemented with three different phytase-containing products at an intended activity of 500 U/kg of diet. The supplemented phytase products were a commercial *A. niger*-derived 3-phytase (PhyA; Finase® P, *EC* 3.1.3.8; AB Vista) and two *E. coli*-derived thermotolerant 6-phytases (PhyE1 (Quantum®) and PhyE2 (Quantum® Blue); *EC* 3.1.3.26; AB Vista). To ensure adequate mixing of each phytase, premixes of each product were prepared by mixing with a small amount of the BD before addition to the treatment diet. Diets were pelleted through a 3-mm die without using steam. The temperature of pellets measured immediately after release from the press ranged between 57°C and 69°C. Representative samples of the diets were taken for analyses of phytase activity, proximate nutrients, DM, Ca, tP, Ti and InsP isomers. The samples were pulverised using a vibrating cup mill (type 6-TOPF; Siebtechnik GmbH) and stored at 4°C until further handling. The experimental diets contained similar concentrations of *myo*-inositol pentakisphosphate ($InsP_5$) and $InsP_6$ (Table 1). The $InsP_6$-P was 57 % of tP in the diets on average and that of $InsP_5$-P was 3 % of tP. Lower InsP isomers were not detected in the diets. The phytase activity of the BD was below the limit of detection, but phytase activities of the supplemented diets ranged between 399 and 467 U/kg of diet.

Animals and management

The study was conducted in the Agricultural Experiment Station of Hohenheim University, location Lindenhöfe in Eningen (Germany). It was approved by the Animal Welfare Commissioner of the University in accordance with the German Welfare Legislation. Birds underwent routine vaccination against coccidiosis, Newcastle disease and infectious bursal disease on 3, 10 and 14 d of age, respectively.

A total of 600 unsexed Ross 308 broilers aged 1 d were obtained from a commercial hatchery (Brüterei Süd GmbH & Co.) and randomly allocated to forty floor pens (approximately 1·5 m × 1·5 m) bedded with wood shavings. Each pen had fifteen birds. The room temperature was 34 and 32°C on days 1 and 2, respectively. Thereafter, the temperature was reduced in steps of 0·5°C per d, reaching 20°C on day 25. Artificial lighting

Table 1. Ingredient composition and analysed characteristics of the experimental diets

	BD	PhyA	PhyE1	PhyE2
Ingredients (g/kg as fed)				
Maize	553			
Solvent-extracted soyabean meal (48 % crude protein)	400			
Soyabean oil	20			
Limestone	13			
Sodium chloride	1			
Choline chloride	2			
Sodium bicarbonate	3			
Mineral mix*	1			
Vitamin mix†	2			
Titanium dioxide	5			
Analysed characteristics of the diets				
DM (g/kg)	895	896	896	895
Crude ash (g/kg)	53	52	53	51
Crude protein (g/kg)	230	231	232	226
Diethyl ether extract (g/kg)	54	53	52	53
Crude fibre (g/kg)	23	21	24	22
Metabolisable energy (calculated) (MJ/kg)	12·5	12·5	12·5	12·5
Phytase activity (U/kg)‡	<50	399§	467	442
Ca (g/kg DM)	7·5	7·6	7·4	7·3
Total P (g/kg DM)	5·2	5·3	5·2	5·2
$InsP_6$-P (g/kg DM)	3·0	3·0	3·0	3·0
Ins(1,2,3,4,6)P_5 (nmol/g DM)	ND	<LOQ	<LOQ	<LOQ
Ins(1,2,3,4,5)P_5 (nmol/g DM)	200	200	300	300
Ins(1,2,4,5,6)P_5 (nmol/g DM)	600	600	600	600
Ins(1,3,4,5,6)P_5 (nmol/g DM)	ND	ND	ND	ND
$InsP_6$ (nmol/g DM)	15 900	16 000	16 200	16 100

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); $InsP_5$, *myo*-inositol pentakisphosphate; ND, not detected; LOQ, limit of quantification.

* P-free: Mineral mix (Gelamin SG 1 Geflügel, GFT MBH), provided per kg of complete diet: 15 mg Cu; 1·6 mg I; 90 mg Fe; 120 mg Mn; 80 mg Zn; 0·5 mg Se; 0·6 mg Co.

† Vitamin mix (Raiffeisen Kraftfutterwerke Süd GmbH), provided per kg of complete diet: 3·6 mg retinol; 75 µg cholecalciferol; 30 mg α-tocopherol; 2·4 mg menadione; 3 mg thiamin; 6 mg riboflavin; 6 mg pyridoxine; 0·03 mg cyanocobalamin; 50 mg nicotinic acid; 14 mg pantothenic acid; 0·1 mg biotin; 1 mg pteroyl(mono)glutamic acid.

‡ Determined at pH 4·5 and 60°C.

§ Determined at pH 5·0 and 37°C.

was provided with an intensity of 10 lux. During the first 2 d, the animal house was illuminated continuously. A lighting regimen of 18 h light and 6 h dark was applied from day 3 onwards. Feed and tap water were available for *ad libitum* consumption. Until day 15, the animals were fed a commercial starter diet containing 1·10 % Ca, 0·55 % tP, 22·0 % crude protein, 6·6 % diethyl ether extract and 12·5 MJ metabolisable energy/kg. On day 16, the birds were weighed, and ten pens of fifteen birds were assigned to each of the four dietary treatments and distributed in a completely randomised block design.

Sampling and analytical methods

At 25 d of age, the animals were asphyxiated by CO_2 exposure and weighed. To standardise feed intake before sampling and thus retention time of feed in the crop, birds were deprived of feed for 1 h. The feed troughs were then moved back into the pens 1 h before the birds were killed, on an individual-pen basis to ensure the same time schedule for all replicates. The first samples were taken 1 h after the beginning of the light period. Samples from five parts of the digestive tract (crop, proventriculus and gizzard (pooled), duodenum and jejunum (pooled), the terminal part of the ileum (defined as the posterior two-thirds of the section between Meckel's diverticulum and 2 cm anterior to the ileo-caeco-colonic junction[23]) and the caeca) were taken. After opening of the abdominal cavity, the total digestive tract was removed except the crop. Digesta of the intestinal segments were gently flushed out with double-distilled water whereas the segments of the anterior digestive tract (crop, proventriculus and gizzard) were cut open and purged. The samples were pooled for all birds from one pen separately for each segment, immediately frozen at –18°C, freeze-dried (type Delta 1-24; Martin Christ Gefriertrocknungsanlagen GmbH) and pulverised as explained for the diets. The ground samples were stored at 4°C until analysis.

Concentrations of proximate nutrients were determined according to the official methods in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten; VDLUFA)[24]. Feed samples were analysed for DM and crude ash (method 3.1), crude protein (method 4.1.1), diethyl ether extract (method 5.1.1) and crude fibre (method 6.1.1). The concentrations of Ca, tP and Ti in diet and digesta samples were determined by a modification of the method of Boguhn *et al.*[25]. In brief, 20 ml of sulfuric acid (18 mol/l) and 2·5 ml of nitric acid (14 mol/l) were added to 0·4 g of sample. Solutions were heated from 100 to 200°C for 30 min in a block digestion system equipped with a system to trap nitrous gases (Behr K 20 L; Behr Labor-Technik GmbH). After cooling to 100°C, 2·5 ml of the nitric acid were added. Following heating from 225 up to 300°C for 75 min and subsequent cooling to room temperature, the solutions were filled with double-distilled water to a volume of 500 ml and filtered. The Ca, P and Ti concentrations of the solutions were measured using an inductively coupled plasma optical emission spectrometer (VISTA PRO; Varian Inc.) at specific wavelengths for each element according to Shastak *et al.*[26].

For the analysis of InsP isomers in diet and digesta samples, 1·0 g of the sample was extracted for 30 min with 10 ml of a solution containing 0·2 M-EDTA and 0·1 M-sodium fluoride (pH = 10) as phytase inhibitor using a rotary shaker. The samples were centrifuged at 12 000 ***g*** for 15 min and the supernatant fraction was removed and preserved on ice. The residue was re-suspended in 5 ml of the EDTA–sodium fluoride solution and extracted again for 30 min. The supernatant fractions of the two extraction steps were then combined. A quantity of 1 ml of the pooled supernatant fraction was centrifuged at 14 000 ***g*** for 15 min and 0·5 ml of the resulting supernatant fraction were filtered through a 0·2 µm cellulose acetate filter (VWR) into a Microcon filter (cut-off 30 kDa) device (Millipore) and centrifuged again at 14 000 ***g*** for 30 min. Throughout the whole extraction procedure, the samples were kept below 6°C. The procedure for caecal samples was slightly different: glass beads (diameter 0·6 mm) were added before extraction. To obtain a clear supernatant fraction for the caecal

matrix, the extracts were centrifuged for 30 min at 12 000 ***g*** and 6°C. Filtrates were analysed by high-performance ion chromatography and UV detection at 290 nm after post-column derivatisation using an ICS-3000 system (Dionex). InsP with different degrees of phosphorylation ($InsP_{3–6}$) and their positional isomers were separated, without enantiomer differentiation, on a Carbo Pac PA 200 column and corresponding guard column. $Fe(NO_3)_3$ solution (1 g/l, $Fe(NO_3)_3.9H_2O$, product no. 103883; Merck KGaA) in $HClO_4$ (20 g/l, product no. 100518; Merck KGaA) was used as reagent for derivatisation according to Philippy & Bland[27]. The elution order of InsP isomers was established using commercial standards if available. $InsP_5$ isomer standards were purchased from Sirius Fine Chemicals. Seven out of nine *myo*-inositol tetrakisphosphate ($InsP_4$) and nine out of twelve *myo*-inositol trisphosphate ($InsP_3$) isomer standards were available from Santa Cruz Biotechnology. One detected peak out of the group of $InsP_4$ isomers could not be attributed but was presumed to be Ins (1,2,4,6)P_4 by comparison with the elution order of Chen & Li[28], who used similar chromatographical conditions. A clear identification of the $InsP_3$ isomers present was not possible. However, a peak was detected, corresponding in its retention time to the retention time of Ins(1,3,4)P_3, Ins(1,4,6)P_3, Ins (1,2,6)P_3, Ins(1,4,5)P_3 and Ins(2,4,5)P_3 (out of the available standards), which all coeluted under the conditions used. $InsP_2$ and $InsP_1$ could not be analysed with this method. $InsP_6$ was used for quantification, and correction factors for differences in detector response for $InsP_{3–5}$ were used according to Skoglund *et al.*[29]. The limit of detection was defined for a signal:noise ratio of 3:1 and was 0·1 μmol/g of DM for $InsP_{3–4}$ isomers and 0·05 μmol/g of DM for $InsP_5$ isomers and $InsP_6$. The limit of quantification was defined for a signal: noise ratio of 6:1. A mean for an InsP isomer was calculated only if the isomer was detected in at least five out of the ten samples of one treatment. If the detected value was below the limit of quantification in five or more samples, this was noted as less than the limit of quantification in the tables, and means were not calculated. All samples were analysed in duplicate. The InsP concentration is reported on a DM basis.

Because of differences in the extractability of the phytases used, the phytase activity in the diets was determined under product-specific conditions and expressed as U/kg for all diets. Determination of phytase activity was assayed according to the internal, validated methods of the supplier (BD + PhyA: assay at pH 5·5 and 37°C; BD, BD + PhyE1 and BD + PhyE2: assay at pH 4·5 and 60°C). Both assays were run by Enzyme Services & Consultancy.

Calculations and statistical analysis

Body-weight gain, feed consumption and feed:gain ratio were determined on a pen basis for the period between days 16 and 25. $InsP_6$ hydrolysis and P net absorption in the digestive tract (y) were calculated for each pen based on the ratio of $InsP_6$ or P and Ti according to the generally accepted equation:

$$y(\%) = 100 - 100 \times \left(\frac{\text{Ti in the diet (g/kg DM)}}{\text{Ti in the digesta (g/kg DM)}}\right) \times \left(\frac{\text{InsP}_6 \text{ or P in the digesta (g/kg DM)}}{\text{InsP}_6 \text{or P in the diet (g/kg DM)}}\right)$$

$InsP_6$ hydrolysis was calculated for the crop, duodenum/jejunum, lower ileum and caeca. It was not calculated for the proventriculus/gizzard since this segment clearly contained particles of different sizes, which presumably were of variable retention times and thus were not accurately represented by the marker[30]. P net absorption was calculated in the duodenum/jejunum and lower ileum.

The percentage of $InsP_3$, $\sum InsP_4$ or $\sum InsP_5$ in $\sum InsP_{3–5}$ was calculated for each treatment and segment to investigate the rapidity of $InsP_6$ hydrolysis and the extent to which intermediary products with different degrees of phosphorylation were formed. Because $InsP_{1–2}$ isomers were not determined, $\sum InsP_{3–5}$ was calculated, representing the sum of identified $InsP_6$ hydrolysis products.

Untransformed data are expressed as means with their standard error or the pooled standard error of the mean. Statistical analysis was performed using the MIXED procedure of the software package SAS for Windows (version 9.1.3; SAS Institute Inc.). Before statistical analysis, data that showed non-normal residuals or heterogeneity of variance were log- or square root-transformed. For data expressed as percentages the arc-sine transformation was used. The following statistical model was chosen: $y_{ij} = \mu + r_i + \tau_j + e_{ij}$, where y_{ij} is the ith measurement in the jth treatment, μ is the overall mean, r_i is the ith block (random), τ_j is the effect of the jth treatment (fixed) and e_{ij} is the error term. Statistical significance was evaluated by a one-way ANOVA. Mean separation was computed using Fisher's protected least significant difference test ($P \leq 0·05$) only if the overall F test was significant ($P \leq 0·05$).

Results

The initial body weight was on average 531 g and was similar between treatments ($P > 0·05$). During the 9-d assay period, average body-weight gain, feed consumption and feed:gain ratio were not significantly different between treatments (Table 2).

Table 2. Body-weight (BW) gain, feed consumption (FC) and feed:gain (F:G) ratio of broiler chickens between the ages of 16 and 25 d (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
BW gain (g/d)*	59	58	60	56	1·4
FC (g/d)*	95	94	97	92	1·9
F:G ratio (g/g)*	1·61	1·61	1·62	1·63	0·023

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue.
* The overall *F* test was not significant.

Table 3. *Myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) hydrolysis (%) in different segments of the digestive tract of broiler chickens (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
Crop	9[d]	64[a]	31[c]	44[b]	4·5
Duodenum/jejunum	59	63	65	68	2·8
Lower ileum	74	74	79	82	3·8
Caeca	91[b]	93[b]	95[a]	96[a]	0·7

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue.
[a–d] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.

Myo-*inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) hydrolysis*

In birds fed the BD, low $InsP_6$ hydrolysis was measured in the crop (9 %; Table 3). Average $InsP_6$ hydrolysis in this treatment was 59, 74 and 91 % until the duodenum/jejunum, the lower ileum and the caeca, respectively.

Supplementation of phytase had a significant effect on $InsP_6$ hydrolysis in the crop ($P < 0{\cdot}01$) but not in the duodenum/jejunum and lower ileum. In the crop, supplemented phytases significantly increased $InsP_6$ hydrolysis, and the effect by PhyA (64 %) was significantly higher than by the other phytase treatments (PhyE1: 31 %; PhyE2: 44 %). Until the duodenum/jejunum and the lower ileum, the difference in $InsP_6$ hydrolysis between treatments became lower and statistical difference between treatments disappeared. The average $InsP_6$ hydrolysis was 64 % (duodenum/jejunum) and 77 % (lower ileum). $InsP_6$ hydrolysis up to the caeca was significantly higher with PhyE1 (95 %) and PhyE2 (96 %) compared with the BD (91 %) and PhyA (93 %) ($P < 0{\cdot}01$).

Net absorption of phosphorus

In birds fed the BD, a P net absorption of 34 and 57 % was measured until the duodenum/jejunum and lower ileum (Table 4). Supplementation of phytases caused a significant increase in P net absorption until the duodenum/jejunum (PhyA: 38 %; PhyE1: 38 %; PhyE2: 39 %) ($P = 0{\cdot}04$). P net absorption until the lower ileum tended to be higher with PhyE1 (60 %) and was significantly higher with PhyE2 (64 %) compared with PhyA (56 %) and BD (57 %) ($P = 0{\cdot}03$).

Appearance of inositol phosphate isomers

In the crop digesta of birds fed the BD, an average concentration of 638 and 388 nmol/g DM was detected for Ins $(1,2,4,5,6)P_5$ and Ins $(1,2,3,4,5)P_5$ and lower concentrations were measured for Ins $(1,2,3,4,6)P_5$ and Ins $(1,3,4,5,6)P_5$ (Table 5 and Fig. 1(A)). The only detectable inositol tetrakisphosphate Ins $(1,2,5,6)P_4$ was found in low concentration (141 nmol/g DM), and inositol trisphosphates were not found in the crop in this treatment. About 90 % of $\Sigma InsP_{3-5}$ in the crop was present as $InsP_5$ when the BD was fed. In the proventriculus/gizzard, Ins $(1,2,4,5,6)P_5$ and Ins $(1,2,3,4,5)P_5$ were the only detectable lower InsP (Table 6), and their concentrations were lower than in the crop. In the duodenum/jejunum, the InsP pattern again was more diverse than in the proventriculus/gizzard and different from the crop (Table 7).

The predominant $InsP_5$ isomer changed in the intestinal segments (Fig. 1 (B) and (C)). Ins $(1,2,3,4,5)P_5$ was the predominant $InsP_5$ isomer in the duodenum/jejunum and the subsequent intestinal segments, accompanied by Ins $(1,2,3,4,6)P_5$ and Ins $(1,2,4,5,6)P_5$ (Tables 7–9). In the duodenum/jejunum, high concentrations of Ins $(1,2,3,4)P_4$ (355 nmol/g DM) were noted and it remained the predominant $InsP_4$ isomer in subsequent segments in the BD treatment. In the lower ileum, the same pattern of $InsP_5$ isomers as in the duodenum/jejunum was found whereas within the $InsP_4$ isomers, Ins $(1,2,4,6)P_4$ also appeared (Table 8). The pattern of the $InsP_5$ isomers in the caeca of birds fed the BD was similar to that of the lower ileum, except that Ins $(1,3,4,5,6)P_5$ appeared in relatively low concentrations (75 nmol/g DM) (Table 9). High concentrations of Ins $(1,2,3,4)P_4$ (750 nmol/g DM) were detected in the caeca whereas Ins $(1,2,4,6)P_4$ was not found and $InsP_3$ and traces of Ins $(1,2,5,6)P_4$ appeared.

In the crop digesta of all phytase-containing treatments, the $InsP_5$ pattern was less broad compared with the BD. For the PhyA treatment, the percentage of Ins $(1,2,4,5,6)P_5$ in $\sum InsP_5$ was significantly higher ($P < 0{\cdot}01$) whereas the percentage of Ins $(1,2,3,4,5)P_5$ in $\sum InsP_5$ was significantly lower ($P < 0{\cdot}01$) compared with the three other treatments (Fig. 1 (A)). In

Table 4. Net absorption of phosphorus (%) in segments of the small intestine of broiler chickens (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
Duodenum/jejunum	34[b]	38[a]	38[a]	39[a]	1·2
Lower ileum	57[b]	56[b]	60[a,b]	64[a]	2·1

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue.
[a,b] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.

Table 5. Concentrations of different inositol phosphate (InsP) isomers (nmol/g DM) in the crop digesta (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
$InsP_3$*	ND	4268^{a}	783^{b}	845^{b}	214
Proportion of $InsP_3$ in $\Sigma InsP_{3–5}$		$0{\cdot}69^{a}$	$0{\cdot}15^{b}$	$0{\cdot}13^{b}$	0·018
Ins(1,2,4,6)P_4	ND	ND	ND	ND	
Ins(1,2,3,4)P_4	ND	ND	170	ND	20·8
Ins(1,2,5,6)P_4	141^{c}	922^{c}	2708^{b}	4505^{a}	421
Proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$	$0{\cdot}10^{c}$	$0{\cdot}14^{c}$	$0{\cdot}57^{b}$	$0{\cdot}74^{a}$	0·032
Ins(1,2,3,4,6)P_5	116^{a}	ND	65^{b}	ND	5·1
Ins(1,2,3,4,5)P_5	$388^{a,b}$	101^{c}	475^{a}	355^{b}	23·0
Ins(1,2,4,5,6)P_5	638^{b}	886^{a}	371^{c}	241^{d}	46·2
Ins(1,3,4,5,6)P_5	51	ND	ND	ND	8·6
Proportion of $\Sigma InsP_5$ in $\Sigma InsP_{3–5}$	$0{\cdot}90^{a}$	$0{\cdot}16^{c}$	$0{\cdot}28^{b}$	$0{\cdot}13^{c}$	0·037
$InsP_6$	$14\,491^{a}$	5790^{d}	$10\,922^{b}$	8670^{c}	720

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue; $InsP_3$, *myo*-inositol trisphosphate; ND, not detected (the InsP isomer was not detectable in the majority of samples); $InsP_4$, *myo*-inositol tetrakisphosphate; $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate).
[a–d] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.
* At least one out of the following $InsP_3$ isomers: Ins(1,4,5)P_3, Ins(1,2,6)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3, Ins(1,4,6)P_3.

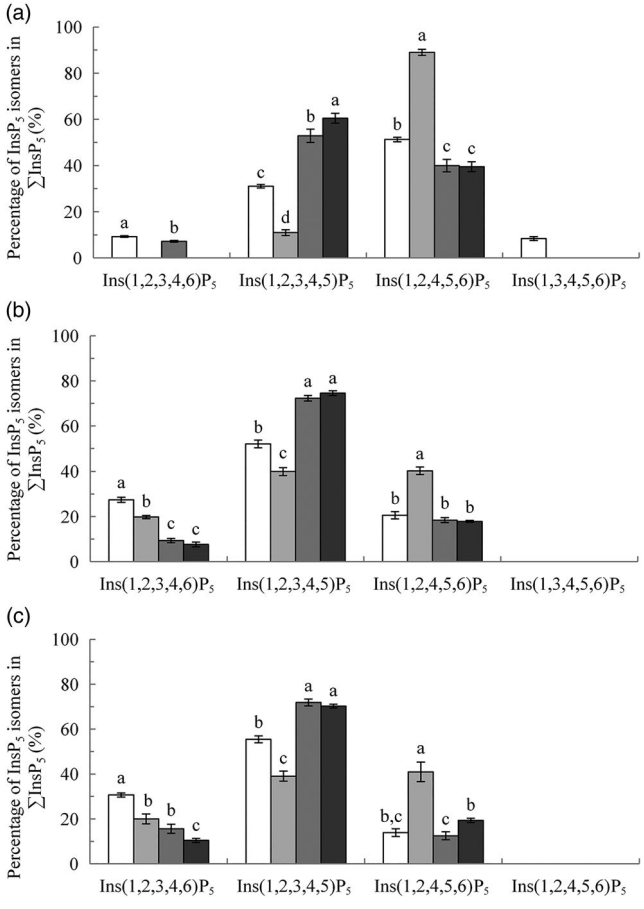

Fig. 1. *Myo*-inositol pentakisphosphate ($InsP_5$) isomers in the crop (A), duodenum/jejunum (B) and ileum (C), expressed as a percentage of $\sum InsP_5$. Values are means, with their standard errors represented by vertical bars. [a–d] Values with unlike letters within an $InsP_5$ isomer were significantly different ($P \leq 0{\cdot}05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant. □, Basal diet (BD); ▒, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P (PhyA); ▒, BD supplemented with *Escherichia coli* 6-phytase, Quantum® (PhyE1); ▓, BD supplemented with *E. coli* 6-phytase, Quantum® Blue (PhyE2).

contrast, for PhyE1 and PhyE2, the percentage of Ins(1,2,3,4,5)P_5 in $\sum InsP_5$ was significantly higher, but the percentage of Ins(1,2,4,5,6)P_5 in $\sum InsP_5$ was significantly lower compared with the two other treatments. Ins(1,2,5,6)P_4 and $InsP_3$ also occurred in the crop in high concentrations for the phytase-containing treatments (Table 5). Ins(1,2,5,6)P_4 appeared for the PhyE1 and PhyE2 treatment in significantly higher concentrations compared with the BD and PhyA ($P < 0{\cdot}01$) and as the only inositol tetrakisphosphate formed by PhyA. $InsP_3$ appeared in significantly higher concentrations for PhyA than PhyE1 and PhyE2 ($P < 0{\cdot}01$). The proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$ was significantly higher for PhyE2 (74 %) than PhyE1 (57 %) and PhyA (14 %) ($P < 0{\cdot}01$), and that of $InsP_3$ in $\Sigma InsP_{3–5}$ was significantly higher for PhyA (69 %) compared with PhyE1 (15 %) and PhyE2 (13 %) ($P < 0{\cdot}01$) in the crop. In the proventriculus/gizzard, the $InsP_5$ pattern of the phytase-containing treatments was similar to that of the crop except Ins(1,2,3,4,6)P_5 was not seen in the proventriculus/gizzard (Table 6). Ins(1,2,5,6)P_4 and $InsP_3$ were detected in significantly lower concentrations for PhyA compared with PhyE1 and PhyE2 ($P < 0{\cdot}01$ for both). A higher proportion of $\Sigma InsP_5$ in $\Sigma InsP_{3–5}$ and a lower proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$ were identified for PhyA compared with PhyE1 and PhyE2 ($P < 0{\cdot}01$).

In the segments of the small intestine, almost the same InsP isomers were detected in all treatments, except Ins(1,2,5,6)P_4, which occurred only in the phytase treatments (Tables 7 and 8). In the duodenum/jejunum, Ins(1,2,3,4,6)P_5 appeared for the first time for PhyA and PhyE2 and again appeared for PhyE1. Ins(1,2,3,4)P_4 was the predominating $InsP_4$ isomer for all treatments. The concentrations of Ins(1,2,3,4,5)P_5 in the duodenum/jejunum were similar for the BD and the PhyA, but significantly higher for the PhyE1 and PhyE2 treatments ($P < 0{\cdot}01$) (Table 7). Ins(1,2,4,5,6)P_5 was detected in significantly higher concentrations for the PhyA compared with the other treatments ($P < 0{\cdot}01$). For all phytase treatments, a significantly lower proportion of Ins(1,2,3,4,6)P_5 in $\Sigma InsP_5$ was determined

Table 6. Concentrations of different inositol phosphate (InsP) isomers (nmol/g DM) in the proventriculus/gizzard digesta (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
$InsP_3$*	ND	105[c]	348[b]	517[a]	54·2
Proportion of $InsP_3$ in $\Sigma InsP_{3–5}$		0·10	0·09	0·11	0·012
Ins(1,2,4,6)P_4	ND	ND	ND	ND	
Ins(1,2,3,4)P_4	ND	ND	<LOQ	ND	
Ins(1,2,5,6)P_4	ND	254[c]	2457[b]	3537[a]	211
Proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$		0·22[c]	0·67[b]	0·79[a]	0·017
Ins(1,2,3,4,6)P_5	ND	ND	ND	ND	
Ins(1,2,3,4,5)P_5	92[c]	107[c]	659[a]	295[b]	30·6
Ins(1,2,4,5,6)P_5	188[b]	618[a]	155[b]	80[c]	28·0
Ins(1,3,4,5,6)P_5	ND	ND	ND	ND	
Proportion of $\Sigma InsP_5$ in $\Sigma InsP_{3–5}$	1·00[a]	0·68[b]	0·24[c]	0·09[d]	0·020
$InsP_6$	6877[a]	4968[b]	2219[c]	739[d]	196

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue; $InsP_3$, *myo*-inositol trisphosphate; ND, not detected (the InsP isomer was not detectable in the majority of samples); $InsP_4$, *myo*-inositol tetrakisphosphate; LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples); $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate).
[a–d] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0·05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.
* At least one out of the following $InsP_3$ isomers: Ins(1,4,5)P_3, Ins(1,2,6)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3, Ins(1,4,6)P_3.

compared with the BD in the duodenum/jejunum (Fig. 1 (B)) and lower ileum (Fig. 1 (C)) ($P < 0·01$). In the lower ileum, the significant difference in Ins(1,2,4,5,6)P_5 concentrations persisted ($P < 0·01$) whereas the difference in Ins (1,2,3,4,5)P_5 concentrations lost significance (Table 8). $InsP_3$ was not detectable in any of the treatments in the small intestine.

In the caeca, the differences in InsP patterns between the phytase-containing diets and the BD were less distinct than in other sections. Concentrations of Ins(1,2,3,4,6)P_5 and Ins (1,2,3,4)P_4 were lower ($P < 0·01$) and concentrations of $InsP_3$ ($P = 0·03$) and Ins(1,2,5,6)P_4 ($P = 0·04$) partially higher when phytases were supplemented, especially for PhyE2 (Table 9). The specific InsP isomers of the supplemented phytases were still present in the caeca. The predominating $InsP_5$ isomer was Ins(1,2,3,4,5)P_5 for all phytase treatments. For the PhyA treatment, significantly higher concentrations of Ins (1,2,4,5,6)P_5 were measured compared with the other treatments ($P < 0·01$).

Discussion

Myo-*inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) hydrolysis and net absorption of phosphorus*

In agreement with previous studies that used low-Ca and low-P diets[11–13] we found a high rate of $InsP_6$ hydrolysis (76 %) and P net absorption (57 %) in the lower ileum. This raises the question of the origin of phytase responsible for this hydrolysis. We found that without supplemented phytase, the majority of $InsP_6$ hydrolysis occurred by the end of the duodenum/jejunum, but hydrolysis still continued in the ileum and caeca. In the small intestine of broilers, the greatest endogenous mucosa phytase activity was found in the

Table 7. Concentrations of different inositol phosphate (InsP) isomers (nmol/g DM) in the duodenal/jejunal digesta (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
$InsP_3$*	ND	ND	ND	ND	
Ins(1,2,4,6)P_4	ND	ND	ND	ND	
Ins(1,2,3,4)P_4	355	<LOQ	303	265	75·1
Ins(1,2,5,6)P_4	ND	<LOQ	123[b]	234[a]	44·9
Proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$	0·28		0·26	0·27	0·025
Ins(1,2,3,4,6)P_5	207[a]	184[a]	110[b]	99[b]	16·7
Ins(1,2,3,4,5)P_5	399[b]	376[b]	864[a]	957[a]	111
Ins(1,2,4,5,6)P_5	148[c]	379[a]	214[b,c]	232[b]	31·7
Ins(1,3,4,5,6)P_5	ND	ND	ND	ND	
Proportion of $\Sigma InsP_5$ in $\Sigma InsP_{3–5}$	0·72[b]	1·00[a]	0·74[b]	0·73[b]	0·022
$InsP_6$	13 392	12 664	11 812	10 701	1076

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue; $InsP_3$, *myo*-inositol trisphosphate; ND, not detected (the InsP isomer was not detectable in the majority of samples); $InsP_4$, *myo*-inositol tetrakisphosphate; LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples); $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate).
[a,b,c] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0·05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.
* At least one out of the following $InsP_3$ isomers: Ins(1,4,5)P_3, Ins(1,2,6)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3, Ins(1,4,6)P_3.

Table 8. Concentrations of different inositol phosphate (InsP) isomers (nmol/g DM) in the digesta of the lower ileum (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
$InsP_3$*	ND	ND	ND	ND	
$Ins(1,2,4,6)P_4$	121	ND	<LOQ	<LOQ	30·5
$Ins(1,2,3,4)P_4$	309	248	282	216	69·3
$Ins(1,2,5,6)P_4$	ND	275	177	192	61·4
Proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$	0·34	0·31	0·31	0·31	0·036
$Ins(1,2,3,4,6)P_5$	231[a]	209[a]	149[a,b]	106[b]	36·0
$Ins(1,2,3,4,5)P_5$	422	410	776	735	156
$Ins(1,2,4,5,6)P_5$	110[b]	463[a]	163[b]	195[b]	51·5
$Ins(1,3,4,5,6)P_5$	ND	ND	ND	ND	
Proportion of $\Sigma InsP_5$ in $\Sigma InsP_{3–5}$	0·66	0·69	0·69	0·69	0·036
$InsP_6$	11 575	12 348	9965	8913	2089

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue; $InsP_3$, *myo*-inositol trisphosphate; ND, not detected (the InsP isomer was not detectable in the majority of samples); $InsP_4$, *myo*-inositol tetrakisphosphate; LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples); $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate).

[a,b] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0·05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.

* At least one out of the following $InsP_3$ isomers: $Ins(1,4,5)P_3$, $Ins(1,2,6)P_3$, $Ins(2,4,5)P_3$, $Ins(1,3,4)P_3$, $Ins(1,4,6)P_3$.

duodenum[8,10]. Phytate-degrading activity has also been reported for different lactic acid bacteria isolated from chicken intestine[31]. Thus, intestinal $InsP_6$ hydrolysis was the result of a combination of endogenous and microbiota phytase, with as-yet-unknown contributions from each source. When phytase activity in different segments of the digestive tract was compared, the highest activity was found in the caeca[32] and Kerr *et al.*[14] detected higher concentrations of $InsP_6$ in the caeca of gnotobiotic compared with conventional broilers. In line with this observation, caecal $InsP_6$ hydrolysis determined in the present study was greater than 90 %. It should be noted that retrograde movement of digesta and micro-organisms has been described for all segments of the digestive tract in broilers[33] and it cannot be ruled out that this affected concentrations of InsP isomers, P and Ti anterior to the caeca.

With supplemented phytase, the crop and the proventriculus/gizzard were the main sites of $InsP_6$ hydrolysis in the present study. The differences found between the supplemented phytases in these segments might be related to differences in enzyme kinetics, pH or resistance against gastrointestinal proteases. A higher temperature optimum was reported for *E. coli* compared with *Aspergillus* phytases[34,35]. At approximate body temperature (42°C), *Aspergillus* phytases show an activity of 85 % of the maximum whereas the activity of some *E. coli* phytases is reduced to 60 % of the *in vitro* analysed maximum[34]. Furthermore, *E. coli* phytases are more resistant than *Aspergillus* phytases against pepsin and pancreatin and show a higher activity at pH 3, which is close to the pH in the proventriculus/gizzard[35–37]. This might explain why the differences between phytases noted in the crop disappeared in the duodenum/jejunum. Moreover, a residual activity of 93 and 60 % has been found for an *E. coli* and an *Aspergillus* phytase, respectively, after incubation in digesta of the proventriculus[35].

Table 9. Concentrations of different inositol phosphate (InsP) isomers (nmol/g DM) in the caecal digesta (Mean values and pooled standard errors; ten pens per treatment with fifteen birds per pen)

	BD	PhyA	PhyE1	PhyE2	Pooled SEM
$InsP_3$*	217[b]	275[b]	355[a,b]	470[a]	59·4
Proportion of $InsP_3$ in $\Sigma InsP_{3–5}$	0·09[b]	0·13[b]	0·19[b]	0·30[a]	0·020
$Ins(1,2,4,6)P_4$	ND	ND	ND	ND	
$Ins(1,2,3,4)P_4$	750[a]	484[b]	422[b]	284[b]	86·1
$Ins(1,2,5,6)P_4$	<LOQ	309[a]	190[b]	318[a]	38·9
Proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$	0·34[b]	0·41[a]	0·36[b]	0·39[a,b]	0·015
$Ins(1,2,3,4,6)P_5$	365[a]	186[b]	88[b]	ND	43·8
$Ins(1,2,3,4,5)P_5$	578[a]	324[b]	436[a,b]	308[b]	63·7
$Ins(1,2,4,5,6)P_5$	119[b]	302[a]	96[b]	82[b]	18·8
$Ins(1,3,4,5,6)P_5$	75	85	86	85	7·5
Proportion of $\Sigma InsP_5$ in $\Sigma InsP_{3–5}$	0·57[a]	0·47[b]	0·45[b]	0·31[c]	0·023
$InsP_6$	6751[a]	4494[b]	3648[b,c]	2255[c]	544

BD, basal diet; PhyA, BD supplemented with *Aspergillus niger* 3-phytase, Finase® P; PhyE1, BD supplemented with *Escherichia coli* 6-phytase, Quantum®; PhyE2, BD supplemented with *E. coli* 6-phytase, Quantum® Blue; $InsP_3$, *myo*-inositol trisphosphate; $InsP_4$, *myo*-inositol tetrakisphosphate; ND, not detected (the InsP isomer was not detectable in the majority of samples); LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples); $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate).

[a,b,c] Mean values in a row with unlike superscript letters were significantly different ($P \leq 0·05$; Fisher's protected least significant difference test). Mean separation was only computed if the overall *F* test was significant.

* At least one out of the following $InsP_3$ isomers: $Ins(1,4,5)P_3$, $Ins(1,2,6)P_3$, $Ins(2,4,5)P_3$, $Ins(1,3,4)P_3$, $Ins(1,4,6)P_3$.

Appearance of inositol phosphate isomers: basal diet

Past experiments in poultry have focused on the analysis of $InsP_6$ and rarely considered clarification of the location of $InsP_6$ hydrolysis[14,32,38]. A few studies have investigated the sum of $InsP_5$, $InsP_4$ and $InsP_3$ isomers in digesta samples of poultry, without differentiation among positional isomers[39–41]. The authors are not aware of any published study investigating positional InsP isomers in the digestive tract of broilers, which led us to making this one of our objectives.

When the BD was fed, the dominating $InsP_5$ isomers found in the crop were Ins(1,2,4,5,6)P_5 and Ins(1,2,3,4,5)P_5. Ins (1,2,3,4,6)P_5 and Ins(1,3,4,5,6)P_5 that occurred in concentrations close to the limit of detection may have originated from the diet (Table 1). The changing pattern of $InsP_5$ isomers (Fig. 1 (A)) in the crop compared with the diet affirm $InsP_6$ hydrolysis in the crop, as does the additional occurrence of $InsP_4$. The appearance of Ins(1,2,4,5,6)P_5 might have been caused by residual intrinsic soyabean phytase, which is a 3-phytase, that withstood exposure to heat in the desolventiser–toaster and was below the limit of detection in the feed. However, this could also have been caused by microbial phytases because 3-phytases are primarily found in fungi (*A. niger*, *A. terreus*, *A. fumigatus*, *Neurospora crassa*), yeasts (*Saccharomyces castellii*, *Saccharomyces cerevisiae*) and bacteria (*Selenomonas ruminantium*, *Selenomonas lacticifex*, *Megasphaera elsdenii*, *Klebsiella terrigena*, *Pantoea agglomerans*, *Pseudomonas syringae*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*). Phytases of plant origin are primarily classified as 4-/6-phytases but 6-phytases were also found for specific bacteria such as *E. coli*, *Peniophora lycii* and *Bifidobacterium pseudocatenulatum*[7,42,43]. Therefore, it remains open whether in the present study Ins(1,2,3,4,5)P_5 was formed by plant or microbiota phytase. Ins(1,2,5,6)P_4 (perhaps co-eluted with Ins(2,3,4,5)P_4) measured in the crop could have been formed by 3- or 6-phytases[5,7]. A formation by other phosphatases which further degraded Ins(1,2,4,5,6)P_5 or Ins(1,2,3,4,5)P_5 to Ins(1,2,5,6)P_4 also is possible.

The fact that there was so little $InsP_5$ in the proventriculus/gizzard suggests that when no phytase is added there is very little $InsP_6$ hydrolysis in that acid environment. Some remaining intrinsic plant phytase may rapidly be inactivated at low pH values and in the presence of pepsin and pancreatin[44–47]. The lack of $InsP_{3–4}$ isomers and the dominance of $InsP_5$ isomers in the proventriculus/gizzard further indicate that $InsP_5$ hydrolysis is less rapidly advanced. However, InsP and their complexes are soluble under acidic conditions and mineral chelates of lower InsPs are more soluble. Hence fast breakdown of $InsP_{3–4}$ also could have happened in the proventriculus/gizzard.

Following passage through the acid phase of the proventriculus/gizzard, a higher accessibility of phytate in the posterior segments can be assumed. In the duodenum/jejunum, a greater range of InsP isomers compared with those found in the stomach was found for the BD treatment, showing intense hydrolysis of $InsP_6$ in this segment. This increase probably was the result of substrate-induced $InsP_6$ hydrolysis by microbiota or endogenous mucosa phytase. Phytate-induced phytase production was reported for several bacteria[48–50]. The results of Schlemmer *et al.*[16] showed substrate dependence of microbiota phytase activity in the colon of pigs. For mucosal phytase, increased activity with dietary phytic acid was also reported in rats[51]. In addition to an alteration of $InsP_6$ hydrolysis and diversity of InsP isomers, the InsP pattern changed between the anterior and intestinal segments of the digestive tract (Fig. 1). This change in InsP pattern suggests the involvement of phosphatases of different origin in different segments, with 3- and 6-phytases dominating in the crop and 6- and 5-phytases dominating in the intestinal segments. Ins(1,2,3,4,5)P_5 can be formed by bacterial 6-phytase of the intestinal microbiota, for example, an *E. coli* 6-phytase. Ins (1,2,3,4)P_4 first appeared in the duodenum/jejunum and was the dominating $InsP_4$ isomer in all intestinal segments when the BD was fed. It might have been a hydrolysis product of a 5-phytase because the majority of phytases continue dephosphorylation adjacent to a free hydroxyl group. Ins(1,2,3,4)P_4 (perhaps co-eluted with Ins(1,2,3,6)P_4) was also characterised as a hydrolysis product of a 5-phytase in lily pollen and *Selenomonas ruminantium* subsp. *lactilytica*[52,53]. However, as Ins(1,2,3,4)P_4 was also detected as a minor hydrolysis product of specific 6-phytases, it principally could have been formed by both 5- and 6-phytases. In addition, the involvement of other phosphatases which further degraded $InsP_5$ cannot be ruled out. Because 5-phytase is described only for *Selenomonas ruminantium* subsp. *lactilytica*[53], lily pollen and *Bifidobacterium pseudocatenulatum*[43], its origin in the intestine of broilers was unexpected. Human gut-isolated *Bifidobacterium pseudocatenulatum* initiates $InsP_6$ hydrolysis at the C-6 and C-5 position of the *myo*-inositol ring and proceeds via Ins(1,2,3,4)P_4[43], but the authors are not aware of any study that found this species of *Bifidobacterium* in broilers.

For the caeca, the broad pattern of InsP isomers is an indication of a highly diverse microbial population likewise producing several phytate-degrading enzymes. From bacteria occurring in the chicken digestive tract, phytate-degrading activity has been described for *Lactobacillus* spp.[31], *Enterobacter* spp.[54], *E. coli*[7], *Klebsiella pneumoniae*[55], *Bacillus* spp.[56], *Bifidobacterium* spp.[57] and *Pseudomonas aeruginosa*[58]. Differences between the InsP pattern of the intestinal segments might be caused by the differing microbial community composition as described by Lu *et al.*[59] and coupled with the variations in activity of the endogenous phytase. Ins(1,3,4,5,6)P_5, which appeared in the caeca, indicates the activity of a phosphatase in the caeca that initiates hydrolysis at the C-2 position of the inositol ring. If this was not undegraded Ins (1,3,4,5,6)P_5 from the feed then this finding contradicts the general assumption that phytate-degrading enzymes are unable to cleave the axial phosphate group of the *myo*-inositol ring.

Appearance of inositol phosphate isomers: phytase treatments

The second objective of the present study was to investigate the $InsP_6$ degradation pattern of different phytase supplements and their effectiveness in releasing phosphate in different

segments of the digestive tract. For the PhyA treatment, Ins (1,2,4,5,6)P_5 was the predominant $InsP_5$ isomer whereas Ins (1,2,3,4,5)P_5 was predominant for the PhyE1 and PhyE2 treatments. This shows, for the first time, that in the crop of broilers the patterns are very similar to the *in vitro* pattern of hydrolysis of these 3- and 6-phytases, initiating $InsP_6$ hydrolysis at the D-3 (L-1) and D-6 (L-4) positions of the inositol ring[5,7]. Ins(1,2,5,6)P_4 (perhaps co-eluted with Ins(2,3,4,5)P_4) and $InsP_3$, both of which were present in high concentrations for the phytase treatments, are the two other main hydrolysis products of the three phytases. This pattern conforms with *in vitro* results that showed D-Ins(2,3,4,5)P_4 as a hydrolysis product of *E. coli* and D-Ins(1,2,5,6)P_4 of *Aspergillus* phytase[5,7]. The proportion of $\Sigma InsP_4$ in $\Sigma InsP_{3–5}$ was higher for PhyE1 and PhyE2 compared with PhyA whereas the proportion of $InsP_3$ in $\Sigma InsP_{3–5}$ was higher for PhyA in the crop. Accumulation of *myo*-inositol tris- and bisphosphates following hydrolysis by *A. niger* phytase has already been shown *in vitro*[60]. In contrast, a fast progression from $InsP_5$ to $InsP_4$ is expected for *E. coli*, but $InsP_4$ accumulated. This result corresponds to *in vitro* findings in which $InsP_4$ accumulated during $InsP_6$ hydrolysis by *E. coli* phytase and was later slowly hydrolysed to $InsP_3$[6]. Because the lower-molecular-weight esters of InsPs have a lower mineral-binding strength than $InsP_6$ or $InsP_5$[61], the solubility of these esters in the small intestine will be improved, allowing access to them by the endogenous phytase/phosphatases[62].

In the duodenum/jejunum and to some extent also in the ileum, the $InsP_5$ and $InsP_4$ isomers specifically formed by the respective supplemented phytases were present in higher concentrations compared with both the BD and the other phytase treatments. Thus, further activity of the enzymes can be assumed in these intestinal segments. However, the proportion of Ins(1,2,3,4,6)P_5 in $\Sigma InsP_5$ was significantly lower, and concentrations of Ins(1,2,3,4)P_4 tended to be lower for the phytase treatments compared with the BD treatment in the duodenum/jejunum and lower ileum. Supplementation of phytase tends to reduce lactic acid bacterial count and significantly reduce *E. coli* count in the ileal digesta of broilers[63], and, as mentioned earlier, both bacterial groups were suspected to be involved in phytate degradation. Aydin *et al.*[63] speculated that the decrease is related to a possible reduction in the quantity of substrate available to the intestinal microbiota. If there was a reduction of lactic acid bacteria (for example, *Bifidobacteria*) this might explain the decrease in 5-phytase activity for all phytase-containing treatments whereas a reduction of *E. coli* bacteria might explain the decrease in 6-phytase activity for PhyA. Furthermore, a reduction in intestinal mucosal phytase activity has been reported when chickens were supplemented with phytase[10]. Thus, a decrease in activity of endogenous mucosal phytase might also have contributed to different InsP pattern between the phytase and the BD treatments in the small intestine. In the lower ileum, the significant differences between treatments in Ins(1,2,4,5,6)P_5 concentration persisted, but the differences in Ins(1,2,3,4,5)P_5 concentration did not. An explanation could be that the activity of supplemented phytases was significantly reduced at this point due to increasing pH and/or proteolytic degradation. Igbasan *et al.*[35] detected a residual activity of 60 and 55 % for an *Aspergillus* and 87 and 80 % for an *E. coli* phytase in the jejunal and ileal digesta, respectively. Intestinal phosphatases might continue to hydrolyse $InsP_{3–5}$ isomers formed by the supplemented PhyE1 and PhyE2, but $InsP_5$ isomers formed by PhyA seemed to be less degradable by intestinal phosphatases. In this regard, Yu *et al.*[64] showed that Ins (1,2,4,5,6)P_5 was a more potent aggregator of protein at low pH compared with Ins(1,2,3,4,5)P_5 which probably would reduce susceptibility of this isomer to phosphatase activity in the intestine.

Conclusions

We conclude that broilers and their microbiota have a high capacity to hydrolyse $InsP_6$ in the intestine. The differentiation between $InsP_6$ hydrolysis products of endogenous or microbiota phytases and their contribution to $InsP_6$ hydrolysis in different segments still requires experimental work. Phytase supplements are more effective in the anterior than in the intestinal segments of the digestive tract, supporting *in vitro* properties. The main $InsP_6$ degradation products of *Aspergillus* and *E. coli* phytases as determined from *in vitro* studies are also formed in the crop and proventriculus/gizzard of broilers. Differences in $InsP_6$ hydrolysis between PhyE1 and PhyE2 compared with PhyA existing in the crop disappeared until the ileum. $InsP_4$ accumulated in the crop when PhyE1 and PhyE2 were used. However, $InsP_3$ accumulated when PhyA was used. It became apparent that the hydrolytic cleavage of the first phosphate group is not the only limiting step in phytate degradation in broilers.

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The authors' contributions are as follows: E. Z. designed and performed the experiments, conducted parts of the chemical analysis, analysed data and drafted the manuscript. M. S. established and optimised the high-performance ion chromatography method and contributed to data presentation and interpretation. I. K. contributed to experimental design and data interpretation. M. R. contributed to all stages of the work, especially in the design, critical reflection on and interpretation of results, and preparation of the manuscript. All authors were involved in the discussion of the results and in editing of the manuscript.

The authors declare that there are no conflicts of interest.

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MANUSCRIPT 2:

Effects of the composition of the basal diet on the evaluation of mineral phosphorus sources and interactions with phytate hydrolysis in broilers

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Abstract

The objective was to determine the availability of P from mineral phosphate sources by using different basal diets in broilers. The investigated criteria were P retention, prececal (pc) P digestibility and pc *myo*-inositol phosphate (InsP) degradation. In experiment 1, semi-synthetic and corn-soybean meal-based basal diets were used. Corn-based and wheat-based basal diets were used in experiment 2. Anhydrous monosodium phosphate (MSP_a) or monocalcium phosphate monohydrate (MCP_h) was supplemented to increment the P concentration by 0.05, 0.10, and 0.15% or by 0.075 and 0.150% in experiments 1 and 2, respectively. Titanium dioxide was used as an indigestible marker. In experiment 1, retention was measured based on total excreta collection from 20 to 24 d of age using 7 replicated birds per diet. In experiment 2, digesta from the terminal ileum was collected from 22-d-old broilers penned in groups of 19 with 5 replicated pens per diet. In experiment 1, the P retention response to supplemented MSP_a did not differ between the 2 basal diets. The response in pc P digestibility to MCP_h supplements also did not differ between the 2 basal diets in experiment 2, as calculated by linear regression analysis. In birds fed the basal diets without a mineral P supplement $InsP_6$ hydrolysis measured on both the excreta and pc levels was high. In both experiments mineral P supplementation significantly reduced ($P < 0.05$) $InsP_6$ hydrolysis from the InsP-containing diets. Thus, the evaluation of the supplemented mineral P source was not affected by the choice of the basal diet. However, calculated values for the use of mineral P sources must be adjusted for the decline in InsP hydrolysis they cause in the respective basal diet.

MANUSCRIPT 3:

Interactions between supplemented mineral phosphorus and phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers

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Abstract

The objectives of this study were to investigate the effects of supplements of mineral phosphorus (P) and phytase as well as their interactions on phytate hydrolysis and the presence of inositol phosphate isomers (InsPs) in the small intestine of broilers. Fifteen-day old broilers were assigned to 48 pens of 20 broilers each (n = 8 pens/treatment). Two low-P corn-soybean meal-based diets without (BD−; 4.4 g P/kg dry matter) or with monocalcium phosphate (MCP; BD+; 5.2 g P/kg dry matter) were supplied without or with added phytase at 500 or 12,500 FTU/kg. On d 24, digesta from the duodenum/jejunum and lower ileum was pooled per segment on a by-pen basis, freeze-dried, and analyzed for P, InsPs, and the marker TiO_2. Another 180 broilers (n = 6 pens/treatment, 10 birds each) were fed the 3 BD+ diets from d 1 to 21 to assess the influence of supplemented phytase on tibia mineralization and strength. Significant interactions between MCP and phytase supplements on *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$) hydrolysis and level of specific lower InsPs were detected. Supplementation with 12,500 FTU/kg phytase resulted in 92% $InsP_6$ hydrolysis and strong degradation of $InsP_5$. Supplementation with 12,500 FTU/kg phytase further resulted in higher P net absorption, affirmed by higher BW gain, tibia strength, and mineralization compared to treatments without or with 500 FTU/kg phytase ($P \leq 0.05$). MCP supplementation reduced the degradation of $InsP_6$ and specific lower InsPs in birds fed diets without or with 500 FTU/kg of phytase ($P \leq 0.05$), but did not reduce $InsP_6$ hydrolysis or degradation of $InsP_5$ at the high phytase dose. It can be concluded that effects of added MCP on phytase efficacy depend on the dose of supplemented phytase. The detected differences in the concentrations of lower InsPs indicated that the initial step of $InsP_6$ hydrolysis is not the only catabolic step that is influenced by MCP or phytase levels.

MANUSCRIPT 4:

Effect of diets containing enzyme supplements and microwave-treated or untreated wheat on inositol phosphates in the small intestine of broilers

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Abstract

The objective of this study was to investigate the effect of diets containing microwave-treated wheat and supplemental enzymes on the hydrolysis of *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$) and the presence of inositol phosphate isomers (InsPs) in the small intestine of broilers. Sixteen-day-old broilers were assigned to 48 pens of 15 broilers each (n = 8 pens per treatment) in a 2 × 3 factorial arrangement of treatments. We fed a wheat-soybean meal diet that was low in phosphorus (4.8 g/kg dry matter) and that contained either microwave-treated (BD_{TW}) or untreated (BD_{UTW}) ground wheat. Diets were used without an enzyme supplement or after supplementation with an *E. coli*-derived phytase, alone or in combination with a xylanase. On d 23, samples of digesta from the duodenum/jejunum and lower ileum were pooled per segment on a by-pen basis, freeze-dried, and analyzed for P, InsPs, and the marker TiO_2. Microwave treatment affected dietary intrinsic phytase activity (BD_{UTW}: 623 U/kg; BD_{TW}: 121 U/kg). In the ileum, significant interactions between microwave treatment and enzyme supplementation were found for $InsP_6$ hydrolysis and P net absorption. In both segments, $InsP_6$ hydrolysis and P net absorption were significantly increased by supplementation of phytase. Supplemental xylanase caused no further increments. The significant interaction on $InsP_6$ hydrolysis in the ileum was due to a higher $InsP_6$ hydrolysis for BD_{TW} (78%) than for BD_{UTW} (69%) in the absence of supplemental phytase. Microwave treatment of wheat had no effect on $InsP_6$ hydrolysis in birds that were fed diets containing phytase. The significant interaction on P net absorption in the ileum was due to reduced P net absorption by microwave treatment in the presence but not absence of supplemental phytase. We conclude that broilers and their gut microbiota have a high potential to hydrolyze $InsP_6$ in the intestine. Microwaving may have disrupted wheat aleurone structures in ways that increased accessibility of $InsP_6$ and may have encouraged higher levels of activity among specific phytases of microbial or endogenous mucosal origin in the lower small intestine. It can further be suggested from the accumulation of $InsP_3$ in the duodenum/jejunum for microwave-treated diets that such treatment reduces the effectiveness of the phosphatases that further degrade $InsP_3$.

MANUSCRIPT 5:

Effect of diets containing enzyme supplements, mineral phosphorus and microwave-treated or untreated wheat on inositol phosphates in the crop of broilers

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Effect of diets containing enzyme supplements, mineral phosphorus and microwave-treated or untreated wheat on inositol phosphates in the crop of broilers

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Running title: Phytate degradation in the crop

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Key words: inositol phosphates; phytate hydrolysis; phytase; xylanase; mineral phosphorus; microwave treatment; broiler; crop;

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Abbreviations: BD-, basal diet without monocalcium phosphate; BD+, basal diet containing monocalcium; BD_{TW}, basal diet containing microwave treated wheat; BD_{UTW}, basal diet containing untreated wheat; DM, dry matter; Enz, enzyme supplementation; InsPs, inositol phosphate isomers; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); LOQ, limit of quantification; MCP, monocalcium phosphate; Mwt, microwave treatment; n.d., not detectable; P, phosphorus; Phy, *E.coli* derived phytase Quantum Blue®; P_i, inorganic phosphate; Ti, titanium; tP, total phosphorus; Xyl, Econase® XT 25

Introduction

Phytate (any salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) or $InsP_6$) represents the major binding form of organic phosphorus (P) in poultry diets. In the digestive tract P is primarily absorbed as orthophosphate. To contribute to the animal's P supply, dietary $InsP_6$-P needs to be cleaved by phytases prior to absorption. Different nutritional factors are known to affect $InsP_6$ hydrolysis in broilers. Besides added microbial phytase, mineral P and factors affecting accessibility or solubility of phytate have been described as influencing factors (Tamin and Angel, 2003; Woyengo et al., 2010; Shastak et al., 2014; Zeller et al., 2015c). Only few studies reported a correlation between the level of intrinsic grain phytase activity and P retention, and thus, indicated an effect of intrinsic plant phytase activity on $InsP_6$ hydrolysis (Barrier-Guillot et al., 1996; Oloffs et al., 2000). When broilers are fed diets that contain recommended industrial standard doses of phytase additives (500-750 FTU/kg of feed), a considerable proportion of dietary $InsP_6$ is not hydrolyzed during passage through the digestive tract. More recent studies found that inclusion of a multiple of the current industry standard dose may achieve $InsP_6$ hydrolysis of more than 90% in broilers (Shirley and Edwards Jr., 2003; Zeller et al., 2015c). Inorganic phosphate (P_i) can reduce activity of phytases and other phosphatases by end product inhibition (Shieh et al., 1969). Thus, degradation of inositol phosphate isomers (InsPs) by phosphatases occurring in the digestive tract and efficacy of added phytases may be diminished by dietary mineral P. In the small intestine of broilers, inclusion of mineral P and Ca reduced phytase efficacy less when phytase was supplemented far above the current standard dose than at the standard dose (Zeller et al., 2015c). In wheat-based diets, xylanase may synergize with phytase since the aleurone layer, whose cell walls predominantly consist of arabinoxylans, saves the major part of phytate in wheat (O'Dell et al., 1972). Thus, xylanase may increase accessibility of phytate for phytases by the hydrolysis of arabinoxylans.

Avian species possess a unique organ, the crop. In theory, pH, which ranges between 3.8 and 6.9 (Svihus, 2010), and other conditions in the crop support the action of intrinsic plant and most microbial phytases. With exception of some bacterial and plant phytases, the pH optimum of currently relevant phytases ranges between 4.0 and 6.0 (Greiner, 2010). Before entering the acid proventriculus/gizzard, dietary phytases can develop catalytic activity at higher pH in the crop, where no secretion of proteolytic enzymes occurs and phytate solubility is higher than in the intestine. Thus, information about the effect of different dietary phytases and other dietary factors on InsP degradation in the crop may play a key role in identification of possibilities to increase $InsP_6$ hydrolysis and degradation of lower InsPs in broilers.

Phytases of different origin produce different positional InsPs and may show different kinetics for the various InsPs. It is still unknown whether microbial and intrinsic plant phytase show synergistic or additive effects on InsP degradation in the crop. Moreover, authors are not aware of any study that investigated the effect of very high levels of added phytase and mineral P and their interactions on InsP degradation in the crop.

Therefore, the first objective was to study the separate and interactive effects of microwave treatment in wheat (applied to inactivate intrinsic plant phytase) and phytase supplemented alone or together with xylanase on $InsP_6$ hydrolysis and lower InsPs in the crop of broilers (experiment 1). The second objective was to investigate the effects of supplemented phytase (a standard and a high level that largely exceeded the current standard level), a mineral P supplement, and their interactions on $InsP_6$ hydrolysis and the appearance of InsPs in the crop (experiment 2).

Material and Methods

Two experiments were conducted. Materials and methods of experiment 1 and 2 were described in detail by Zeller et al. (2015a and 2015c, part 1), respectively.

Experimental diets, animals and management

Both experiments employed a 2x3 factorial arrangement of treatments. In experiment 1, dietary treatments were based on two low-P wheat-soybean meal-based diets, one containing microwave treated wheat (BD_{TW}) the other containing untreated wheat (BD_{UTW}). Concentrations of total P (tP) and Ca were calculated to be 4.7 and 7.8 g/kg of dry matter (DM). Diets were fed without or with supplementation of an *E. coli* derived, thermotolerant phytase (Phy, Quantum Blue®, intended activity 500 FTU/kg), alone or in combination with a commercial *T. reesei* derived thermostable endo-1,4-beta-xylanase (Xyl, Econase® XT 25, intended activity 16,000 BXU/kg, both AB Vista, Marlborough, UK). High phytase activity was measured in BD_{UTW} (623 U/kg), whereas BD_{TW} was very low in phytase activity (121 U/kg). Phytase activity of the enzyme supplemented diets ranged between 341 and 389 FTU/kg of diet. Xylanase activity was below the limit of detection in BD_{UTW}, except in BD_{TW} (1,820 FAXU/kg). Xylanase activity in the xylanase supplemented diets ranged between 11,700 and 14,000 FTU/kg of diet.

In experiment 2, two low-P maize-soybean meal-based diets without (BD-) or with monocalcium phosphate (MCP, BD+) were used. Diets were fed without or with supplementation of an *E. coli* derived phytase (Quantum Blue®, EC 3.1.3.26, supplied by AB Vista, Marlborough, UK) at a level of 500 or 12,500 FTU/kg. The tP and Ca concentrations

were calculated to be 4.5 g/kg and 6.5 g/kg of DM in BD- and 5.4 g/kg and 7.9 g/kg of DM, respectively, in BD+. Phytase activity in BD- and BD+ was below the limit of detection and intended phytase activities in the supplemented diets were confirmed by analyses. In both experiments titanium (Ti) dioxide was included as an indigestible marker and diets were pelletized.

In experiment 1, seven hundred and twenty broiler hatchlings were randomly allocated, 15 birds per pen, to 48 pens. In experiment 2, nine hundred and sixty broiler hatchlings were randomly allocated, 20 birds per pen, to 48 pens. Dietary treatments were assigned to the pens (eight pens per treatment) in accordance with a completely randomized block design in the animal house on day 16 and 15 in experiment 1 and 2, respectively. Diets were fed until slaughter at day 23 and 24 in experiment 1 and 2, respectively. In both experiments, diets and tap water were provided for *ad libitum* consumption.

Sampling and analytical methods

To standardise feed intake prior to sampling and thus retention time of feed in the crop, in both experiments, birds were stimulated to eat in the last hour before slaughter by removing feed two hours prior to killing. Feed was provided again one hour before killing. This was done on a pen-by-pen basis to ensure the same time schedule for all pens. In experiment 1, on day 23, all birds were asphyxiated by CO_2 exposure. In experiment 2, on day 24, 15 out of the 20 birds from each pen were randomly selected and stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2, and euthanized via CO_2 asphyxiation. In both experiments, immediately after the slaughter the crop was cut open and purged. Samples from the crop content were pooled for all birds from one pen, immediately frozen at -18°C, freeze-dried, and pulverized using a vibrating cup mill (Type 6-TOPF, Siebtechnik GmbH, Mülheim-Ruhr, Germany). Pulverized samples were stored at 4 °C until analysis. Samples were analyzed for concentrations of InsPs and Ti.

Calculations and statistical analysis

$InsP_6$ hydrolysis was calculated on a by-pen basis according to the following equation:

$$InsP_6 \text{ hydrolysis } (\%) = 100 - 100 \times \left(\frac{\text{Ti in the diet (g/kg DM)}}{\text{Ti in the digesta (g/kg DM)}}\right) \times \left(\frac{InsP_6 \text{ in the digesta (g/kg DM)}}{InsP_6 \text{ in the diet (g/kg DM)}}\right)$$

Non-transformed data were expressed as means of treatments and their pooled SEM. Data were analyzed using the MIXED procedure of the software package SAS for Windows (version 9.1.3, SAS Institute Inc., Cary, NC, USA). Data that showed non-normally distributed residuals or heterogeneity of variance were log or square-root transformed prior to

statistical analysis. For details on the statistical models the reader is referred to the aforementioned manuscripts of Zeller et al. (2015a and 2015c, part 1). Statistical significance was evaluated by a two-way (2×3) analysis of variance ($P \leq 0.05$) (two microwave treatment and three enzyme levels (experiment 1) and two MCP and three phytase levels (experiment 2)). If an effect of interaction was detected ($P \leq 0.05$), differences between treatments were tested using multiple t-tests at a level of significance of $P \leq 0.05$.

Results

Experiment 1

In experiment 1, microwave treatment of wheat significantly reduced and enzyme supplementation significantly increased $InsP_6$ hydrolysis in the crop (Table 1). $InsP_6$ hydrolysis was significantly increased by supplementation of phytase, but it was not further increased by the additional supplementation of xylanase.

Microwave treatment significantly increased concentrations of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 in the crop. Concentrations of Ins(1,2,3,4,6)P_5, Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 were significantly reduced by supplementation of phytase but not further reduced by the additional supplementation of xylanase. A significant interaction between microwave treatment and enzyme supplementation was found for concentration of Ins(1,2,5,6)P_4. Concentrations of Ins(1,2,5,6)P_4 were significantly reduced when wheat was microwave treated in the diets without enzyme supplements. When the diets were supplemented with phytase concentration of Ins(1,2,5,6)P_4 was significantly increased by microwave treatment. Supplementation of phytase had no effect on Ins(1,2,5,6)P_4 concentration when wheat was untreated, but significantly increased Ins(1,2,5,6)P_4 concentration when wheat was microwave treated; an effect that was not altered with additional supplementation of xylanase. Concentrations of Ins(1,2,3,4)P_4 were significantly decreased by microwave treatment and supplementation with phytase, but not further decreased with additional supplementation of xylanase. Concentrations of $InsP_3$ were significantly reduced by microwave treatment.

Experiment 2

In experiment 2, supplementation of 500 FTU/kg phytase significantly increased $InsP_6$ hydrolysis in the crop, an effect that was further and significantly increased after supplementation of 12,500 FTU/kg phytase (up to 77% (BD-) and 80% (BD+)) (Table 2). Inclusion of MCP did not affect $InsP_6$ hydrolysis.

A significant interaction between MCP and phytase supplements was found for concentrations of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5. This interaction was due to increased concentrations of both InsPs when MCP was supplemented to the diets without phytase. Supplementation with 500 FTU/kg phytase resulted in a significant decrease in concentrations of both InsPs; 12,500 FTU/kg phytase caused further significant reduction in concentrations of Ins(1,2,3,4,5)P_5 but not Ins(1,2,4,5,6)P_5. In broilers fed diets with phytase, Ins(1,2,3,4,6)P_5 was only detected in low concentrations when 500 FTU/kg phytase were supplemented to BD-. Concentrations of Ins(1,2,5,6)P_4 were significantly increased by supplementation with phytase; concentrations of Ins(1,2,5,6)P_4 were significantly higher after treatment with 500 FTU/kg phytase compared to 12,500 FTU/kg phytase. $InsP_3$ was detected in high concentrations in the crop content upon supplementation of 500 FTU/kg phytase.

Discussion

Variability of $InsP_6$ hydrolysis in the crop

In experiment 2, $InsP_6$ hydrolysis was negligible when the maize-based diet without detectable phytase activity was fed. Zeller et al. (2015b) similarly reported low $InsP_6$ hydrolysis in the crop of broilers fed a maize-soybean meal-based diet without enzyme supplements. In contrast, $InsP_6$ hydrolysis in the present study was high (59%) when the diet with high intrinsic phytase activity (623 U/kg) was fed in experiment 1. The pH measured in digesta from the crop averaged 5.33 (SD 0.42) (treatment means did not differ significantly, data not shown) (experiment 1). This pH probably supports the activity of wheat phytases, which under *in vitro* conditions showed maximal activity at pH 6.0 (phytase 1) and pH 5.5 (phytase 2) (Nakano et al., 1999). Lower $InsP_6$ hydrolysis in birds fed BD_{TW} (26%) compared to birds fed BD_{UTW} was most likely the consequence of lower activities of intrinsic wheat phytase and other enzymes due to microwave treatment. In line with this observation, 35% and 72% of phytate were hydrolyzed when heat-treated (190 U/kg) and non-heat-treated (720 U/kg) wheat-based diets were soaked for 2 h at 38°C (Carlson and Poulsen, 2002).

In literature, the benefit of intrinsic plant phytase activity for P digestibility in broilers is controversially discussed. More recent studies found no relationship between dietary intrinsic phytase activity and $InsP_6$ hydrolysis in the ileum of broilers (Leytem et al., 2008b; Shastak et al., 2014). Authors concluded that microbiota- and mucosa- associated phytases have a higher influence than intrinsic plant phytases. The results from the present study showed that the action of intrinsic wheat phytase can cause a high $InsP_6$ hydrolysis in the crop. Hence, intrinsic plant phytases can make a high contribution to the overall $InsP_6$ hydrolysis in

broiler's digestive tract. But, $InsP_6$ hydrolysis by microbiota- and mucosa- associated phytases in the small intestine of broilers may compensate for the effects of intrinsic plant phytases measured in the crop if these plant phytases are not present.

Applying 500 or 12,500 FTU/kg of phytase in maize-based diets increased $InsP_6$ hydrolysis up to an average value of 61% or 79% (experiment 2). This is close to the values measured for the same enzyme levels in the ileum of broilers (Zeller et al., 2015c) and shows that the major proportion of $InsP_6$ hydrolysis measured in the ileum of birds fed the maize-based diets supplemented with phytase already occurred in the crop.

Overall, these findings emphasize the high activity of intrinsic plant and added microbial phytases in the crop of broilers. The data further suggest that $InsP_6$ hydrolysis in the crop is predominantly related to phytases contained in the diet rather than phytases of microbial or mucosal origin.

When 500 FTU/kg phytase were supplemented to the wheat-based diets in experiment 1, the increase in $InsP_6$ hydrolysis was similar in birds fed BD_{UTW} (8%) and BD_{TW} (12%). Thus, supplemented phytase acted rather additive and not competitive to wheat phytases. The major part of wheat phytases and phytate is localized in the same grain fraction, the aleurone layer (Peers, 1953; Fretzdorff and Weipert, 1986), which probably promotes the efficacy of wheat phytase in the crop. Perhaps, this fraction was less accessible for the supplemented phytase within the retention time in the crop. The effect of the supplemented phytase on $InsP_6$ hydrolysis was much higher in broilers fed the maize- (experiment 2) compared to the wheat-based diets (experiment 1). This again suggests that accessibility of phytate for the supplemented phytase is relatively low in wheat, whereas it seems to be higher in maize. In part, this may be related to different phytate localization in the kernel and binding forms of phytate. In maize, 90% of phytate is localized in the germ. In wheat, more than 80% of phytate is present in the aleurone layer and outer brans (O'Dell et al., 1972). Thick aleurone cell walls and other surrounding structures may hinder the access of supplemented phytase to phytate in the aleurone layer. Phytate of the germ, other grain fractions and soybean meal may be more accessible and probably was predominantly hydrolyzed by supplemented phytase in the present study. In agreement with this, Blaabjerg et al. (2007) reported that the effect of phytase addition on phytate degradation during *in vitro* incubation was greatest in soybean meal, almost intermediate for wheat/soybean meal diets and not detectable in wheat. The authors suggested a better accessibility of phytate in soybean meal compared to wheat for added phytase (Blaabjerg et al., 2007). It can be concluded that the efficacy of supplemented

phytase in the crop depends on accessibility of phytate which different between grain species and variety.

Additional supplementation of xylanase caused no significant increase in hydrolysis of the probably less accessible $InsP_6$ in wheat. However, it seems to be interesting to note that a trend was detected in broilers fed BD_{TW} that shows an add-on effect of xylanase on $InsP_6$ hydrolysis (10%) to the supplemented phytase (experiment 1). Probably, changes in structures imposed by microwave treatment of wheat increased accessibility of arabinoxylans for xylanase. Following, hydrolysis of arabinoxylans by xylanase could have increased accessibility of phytate. In agreement with this hypothesis, Cowieson et al. (2005) suggested an increased release of previously encapsulated non-starch-polysaccharides from the diet when conditioning temperature was increased above 80°C.

In experiment 2, supplementation of MCP had no effect on $InsP_6$ hydrolysis in the crop irrespective of the level of added phytase. This suggests that the concentrations of liberated InsP-P_i and P_i from MCP were too low to trigger relevant product inhibition and to reduce $InsP_6$ hydrolysis by added phytase. In part, this may be related to a high activity and reaction rate of added phytase under the advantageous conditions in the crop. It also shows that the higher Ca concentrations and Ca:$InsP_6$ ratio in BD+ compared to BD- diets had no effect on $InsP_6$ hydrolysis, and thus, did not reduce phytate solubility in the crop.

InsPs in the presence of low and high intrinsic phytase activities

The supply of P from InsPs depends on further degradation of $InsP_{1\text{-}5}$. Formation of specific lower InsPs in the anterior segments of the digestive tract may be relevant for later solubility and degradation by specific phosphatases in the small intestine. Therefore, another objective was to study the effect of different dietary factors on the appearance of lower InsPs in the crop.

When the maize-based diets without detectable phytase activity were fed (experiment 2), $InsP_5$ isomers found in the crop mainly originated from the diet. However, comparison of the proportions of $InsP_5$ isomers in $\sum InsP_5$ in feed and crop and the appearance of Ins(1,2,5,6)P_4 in low concentrations in the crop point to some degradation of $InsP_6$ probably caused by activity of 6-phytases. InsP(1,2,3,4,5)P_5 and Ins(1,2,5,6)P_4 could have been formed by maize phytases (personal information of Ralf Greiner) although phytase activity in the diets was below the limit of detection and bacterial phytases (Van der Kaay and Van Haastert, 1995; Greiner et al., 2000; Haros et al., 2009) associated with feed or crop microbiota. Ins(1,2,5,6)P_4

may also be formed by feed-associated fungal 3-phytases (Greiner and Carlsson, 2006; Greiner et al., 2009) or other types of phosphatases, which do not dephosphorylate $InsP_6$.

When the wheat-based diets without supplemental phytase were fed (experiment 1), the dominating InsPs found in the crop were Ins(1,2,3,4,5)P_5 accompanied by Ins(1,2,4,5,6)P_5, Ins(1,2,5,6)P_4 accompanied by Ins(1,2,3,4)P_4 and $InsP_3$. *In vitro*, Ins(1,2,3,4,5)P_5, Ins(1,2,5,6)P_4 and Ins(1,2,6)P_3 were the InsPs mainly formed by phytases purified from wheat (Nakano et al., 2000). Ins(1,2,4,5,6)P_5, Ins(1,2,3,4)P_4 and Ins(1,2,3)P_3 were formed to a minor extent (Nakano et al., 2000; Bohn et al., 2007). This showed for the first time that the InsP pattern in the crop of broilers fed wheat-based diets was very similar to the pattern formed by wheat phytases *in vitro*. Microwave treatment of wheat resulted in higher concentrations of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 and lower concentrations of Ins(1,2,5,6)P_4, Ins(1,2,3,4)P_4 and $InsP_3$ in birds fed diets without supplemental phytase. Obviously, degradation of $InsP_5$ to $InsP_4$ and $InsP_3$ was slower when wheat was microwave treated, probably due to reduced activity of phytases and other wheat phosphatases.

InsPs as affected by supplemented phytase

For the maize-based diets supplemented with phytase (experiment 2) the major InsPs found in the crop were Ins(1,2,3,4,5)P_5, Ins(1,2,4,5,6)P_5, Ins(1,2,5,6)P_4 and $InsP_3$. These results confirm findings of a previous study where Ins(1,2,3,4,5)P_5 and Ins(1,2,5,6)P_4 were specified as the main $InsP_{4\text{-}5}$ isomers formed in the crop of broilers by the same phytase product (Zeller et al., 2015b). It also was suggested that $InsP_3$ (Ins(2,4,5)P_3 may elute with the $InsP_3$ peak and was described as a further degradation product of an *E. coli* phytase (Greiner et al., 2000)) was formed by the supplemented phytase in the crop. In respect of Ins(1,2,4,5,6)P_5, it remains open whether it was formed by the supplemental phytase or originated from feed.

When 500 FTU/kg phytase were supplemented to the maize-based diet, concentrations of $InsP_5$ isomers were reduced but $InsP_4$ accumulated in high concentrations. This suggests a fast progression of $InsP_5$ to $InsP_4$, but further degradation of $InsP_4$ and $InsP_3$ proceeded less rapidly (experiment 2). Significantly lower concentrations of Ins(1,2,3,4,5)P_5, Ins(1,2,5,6)P_4 and $InsP_3$ followed supplementation with 12,500 FTU/kg phytase, suggesting more complete degradation of InsPs at the higher phytase dosage, but $InsP_4$ remained the predominant InsP. It can be concluded, that hydrolysis of $InsP_4$ is a limiting step in $InsP_6$ degradation when this phytase is used irrespective of the phytase level used.

For the wheat-based diets supplemented with phytase, it is not clear which proportions of InsPs were formed by wheat and supplemented phytase (experiment 1). However,

supplementation with 500 FTU/kg phytase reduced concentrations of $InsP_5$ isomers regardless of wheat treatment. This affirms a fast progression of $InsP_5$ to $InsP_4$ in the crop when diets are supplemented with the phytase. Degradation of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 proceeded even more rapidly when phytase was supplemented to diets containing the untreated wheat compared with the microwave treated wheat. This points to a possible synergy of the supplemental phytase with wheat phosphatases on degradation of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5. Correspondingly, a significant interaction between microwave treatment and enzyme supplements was found for concentrations of Ins(1,2,5,6)P_4. Supplementation with phytase did not significantly increase concentrations of Ins(1,2,5,6)P_4 in broilers fed BD_{UTW} but did in BD_{TW}. Further, in the presence of supplemented phytase concentrations of $InsP_3$ were significantly higher in the crop of birds fed BD_{UTW} compared to BD_{TW}. Hence, upon phytase supplementation Ins(1,2,5,6)P_4 accumulated in birds fed the diet with low intrinsic phytase activity whereas Ins(1,2,5,6)P_4 was more rapidly degraded to $InsP_3$ when the diet with high intrinsic phytase activity was fed. These results provide the first indication of a synergistic effect of intrinsic wheat phosphatases and the supplemented *E. coli* phytase on degradation of Ins(1,2,5,6)P_4 in the crop of broilers. A joint degradation is also reflected by the fact that the accumulation of Ins(1,2,5,6)P_4 was lower when phytase was supplemented to BD_{TW} (with low phytase activity) (experiment 1) than to the maize-based diet (without detectable phytase activity) (experiment 2). In line with these observations, a possible synergism of the supplemental phytase with wheat phytases in InsP degradation was suggested from *in vitro* results, which showed higher release of *myo*-inositol when phytase was added to wheat- compared to maize-based diets (Zyla et al., 2013).

In contrast, Ins(1,2,3,4)P_4, which was suggested to be formed by wheat phosphatases, occurred in lower concentrations in the presence of supplemented phytase. This may be explained by the lack of the respective precursor InsP of Ins(1,2,3,4)P_4 when supplemented phytase was active. When phytase was supplemented to BD_{TW}, Ins(1,2,3,4)P_4 was not detected which can be attributed to additional inactivation of the respective wheat phosphatases.

Conclusions

A high activity of wheat phytases can cause high $InsP_6$ hydrolysis in the crop. $InsP_6$ hydrolysis by microbiota- and mucosa- associated phytases in the small intestine may compensate for the effects of intrinsic plant phytases in the crop if they are lacking. For supplemented phytase the accessibility of phytate in wheat seems to be lower than in maize,

perhaps due to different storage sites in the grain. We also conclude that the hydrolysis of the first phosphate group on the $InsP_6$ molecule is not the only step in the degradation process that is influenced by dietary factors. Microwave treatment of wheat weakened degradation of $InsP_6$ and $InsP_5$, due to reduction in intrinsic enzyme activity. In addition to a high $InsP_6$ hydrolysis, supplementation of phytase at levels several fold higher than the current industry standard caused stronger degradation of $InsP_{3-5}$ than at the standard level. Degradation of $Ins(1,2,5,6)P_4$ was a limiting step in the breakdown process of $InsP_6$ by the supplemented phytase. However, $Ins(1,2,5,6)P_4$ seemed to be degraded synergistically by intrinsic wheat phosphatases and the supplemented phytase.

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Table 1. $InsP_6$ hydrolysis (%) and concentrations of different InsPs in the digesta of the crop (nmol/g DM) (experiment 1)[1]

	BD_{UTW}			BD_{TW}			Pooled	P-value		
Supplements	0	Phy	PhyXyl	0	Phy	PhyXyl	SEM	Mwt	Enz	Mwt x Enz
$InsP_3$[2]	1,857	2,060	2,015	435	673	849	224	<0.001	0.154	0.324
Ins(1,2,3,4)P_4[3]	1,015	182	202	290	n.d.	n.d.	77	<0.001	<0.001	.
Ins(1,2,5,6)P_4	1,394^{bc}	1,003^{cd}	1,068^{cd}	827^{d}	2,354^{ab}	2,945^{a}	332	0.126	0.009	<0.001
Ins(1,2,3,4,6)P_5[3]	183	82	87	321	101	84	36	0.421	<0.001	0.257
Ins(1,2,3,4,5)P_5[3]	479	205	202	690	353	299	52	0.004	<0.001	0.711
Ins(1,2,4,5,6)P_5[3]	223	190	159	578	369	296	53	0.003	0.006	0.223
Ins(1,3,4,5,6)P_5	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.				
$InsP_6$[4]	5,056	4,528	3,964	9,054	7,198	6,092	780	0.005	0.032	0.164
$InsP_6$ hydrolysis (%)[3]	59	67	70	26	38	48	6.3	0.002	0.031	0.388

[1]Data are given as means and pooled SEM (untransformed data), *n*= 8 pens per treatment with 15 birds per pen. [2]At least one out of the following $InsP_3$ isomers: Ins(1,4,5)P_3, Ins(1,2,6)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3, Ins(1,4,6)P_3.[3]Means were significantly different between 0 and Phy and between 0 and PhyXyl (calculated across basal diets due to missing interactions), P≤0.05. [4]Means were significantly different between 0 and PhyXyl (calculated across basal diets due to missing interactions), P≤0.05. [a–d] Means in the same row without a common superscript are significantly different according to the t-test (P≤0.05). BD_{UTW}, basal diet containing untreated wheat; BD_{TW}, basal diet containing microwave treated wheat; n.d., not detectable (the InsP isomer was not detectable in the majority of samples); LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples);

Table 2. $InsP_6$ hydrolysis (%) and concentrations of different InsPs in the digesta of the crop (nmol/g DM) (experiment 2)[1]

	BD-			BD+			Pooled	P-value		
Phy	0	500	12,500	0	500	12,500	SEM	Phy	MCP	Phy x MCP
$InsP_3$[2]	n.d.	1,362	230	n.d.	1,626	n.d.	115	.	<0.001	0.120
Ins(1,2,5,6)P_4[3]	169	4,766	1274	251	5,230	1001	293	<0.001	0.768	0.472
Ins(1,2,3,4,6)P_5	213	90	n.d.	240	n.d.	n.d.	21	0.002	0.379	.
Ins(1,2,3,4,5)P_5	569[b]	282[c]	102[d]	668[a]	250[c]	95[d]	37	<0.001	0.574	0.034
Ins(1,2,4,5,6)P_5	1,035[b]	241[c]	172[c]	1,186[a]	209[c]	147[c]	46	<0.001	0.502	0.032
Ins(1,3,4,5,6)P_5	93	n.d.	n.d.	81	n.d.	n.d.	20	.	0.651	.
$InsP_6$[3]	14,242	5,755	3,242	13,779	5,123	2,942	850	<0.001	0.630	0.958
$InsP_6$ hydrolysis (%)[3]	-7	60	77	6	62	80	6.0	<0.001	0.379	0.288

[1]Data are given as means per treatment and SEM (untransformed data), *n*= 8 pens per treatment with 15 birds per pen; [2]At least one out of the following $InsP_3$ isomers: Ins(1,2,6)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3; [3]Means were significantly different between all three Phy levels (calculated across basal diets due to missing interactions), $P \leq 0.05$. [a-e]Different superscripts in a row indicate differences between treatment means (multiple t-tests in case of interaction), $P \leq 0.05$; BD-, basal diet without monocalcium phosphate; BD+, basal diet containing monocalcium phosphate; n.d., not detectable (the InsP isomer was not detectable in the majority of samples);

CHAPTER 5

SUMMARY

5 SUMMARY

Phytate (any salt of *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) or $InsP_6$) represents the major binding form of phosphorus (P) in plant seeds. In the digestive tract, availability of P from plant seeds and feedstuffs obtained thereof largely depends on the enzymatic hydrolysis of $InsP_6$ and less phosphorylated inositol phosphate isomers (InsPs). High prices of mineral P supplements and environmental burden linked with excessive P excretion of animals as well as exhaustion of the global rock phosphate stores demand for maximization of phytate-P utilization in animal feeding. The major objective of this thesis was to understand better $InsP_6$ hydrolysis and formation of lower InsPs in different segments of the digestive tract of broilers and how they can be influenced by different dietary factors.

In the first study (Manuscript 1), broilers (n=10 pens per dietary treatment) were fed low-P (5.2 g/kg DM) corn-soybean meal-based diets without (basal diet) or with one of three different phytase supplements (an *Aspergillus* and two *E. coli* derived phytases) from days 16 to 25 of age. $InsP_6$ hydrolysis until the lower ileum (74%) of birds fed the basal diet indicated a high potential of broilers and their gut microbiota to hydrolyse $InsP_6$ in low-P diets. Different InsP pattern in different gut segments suggested the involvement of phosphatases of mucosal or microbial origin. Supplemented phytases significantly increased $InsP_6$ hydrolysis in the crop but not in the lower ileum. Measurements in the crop and proventriculus/gizzard confirmed published *in vitro* degradation pathways of 3- and 6-phytases for the first time in broilers. Presence of $InsP_4$ and $InsP_5$ isomers specifically formed by different supplemented phytases indicated activity of these enzymes still in the small intestine. $InsP_4$ accumulation differed between the 6- and 3-phytases in the anterior segments of the gut.

In the second study (Manuscript 2), effects of supplemental mineral P were studied using different basal diets. Semi-synthetic and corn-soybean meal-based basal diets (experiment 1), or corn-based and wheat-based basal diets were used (experiment 2). Anhydrous monosodium phosphate (MSP_a) or monocalcium phosphate monohydrate (MCP_h) was supplemented to increment the P concentration by 0.05, 0.10, and 0.15% or by 0.075 and 0.150% in experiment 1 and 2, respectively. In experiment 1, total excreta were collected from day 20 to 24 of age (7 replicated birds per diet). In experiment 2, digesta from the terminal ileum was collected when broilers were 22 days old (5 replicated pens per diet, 19 birds per pen). No differences were found in $InsP_6$ hydrolysis between the maize- and wheat-based diets (experiment 2). Mineral P supplements significantly decreased $InsP_6$ hydrolysis from the InsP-containing diets in both experiments. The choice of the basal diet did not affect the

evaluation of the supplemented mineral P sources. This lead to the conclusion that calculated availability values for mineral P sources need to be adjusted for the decline in hydrolysis of InsP contained in the basal diet resulting from the P supplement.

In the third study (Manuscript 3), broilers (20 birds per pen; n=8 pens per treatment) were fed two low-P corn-soybean meal-based diets without (BD-; 4.4 g P/kg DM) or with monocalcium phosphate (MCP) (BD+; 5.2 g P/kg DM) and without or with added phytase at 500 or 12,500 FTU/kg from days 15 to 24 of age. Digesta samples were taken from the duodenum/jejunum and lower ileum. Another 180 broilers (n=6 pens per treatment, 10 birds each) were fed the three BD+ diets from day 1 to 21 of age to assess the influence of supplemented phytase on tibia mineralization and strength. Interactions between MCP and phytase affected $InsP_6$ hydrolysis and the concentrations of specific lower InsPs. Supplementation with 12,500 FTU/kg phytase resulted in 92% prececal $InsP_6$ hydrolysis and strong degradation of $InsP_5$. This resulted in higher P net absorption, affirmed by higher body weight gain, tibia strength, and mineralization compared to treatments without or with 500 FTU/kg of phytase. MCP supplementation reduced $InsP_6$ hydrolysis and the degradation of specific lower InsPs in birds fed diets without phytase or with 500 FTU/kg of phytase, but did not reduce $InsP_6$ hydrolysis or degradation of $InsP_5$ at the high phytase dose. Hence effects of added MCP on phytase efficacy depend on the dose of supplemented phytase.

In the fourth study (Manuscript 4), broilers (15 birds per pen, n=8 pens per treatment) were fed a wheat-soybean meal diet low in P (4.8 g/kg DM) and containing either microwave-treated (BD_{TW}; 121 U/kg of phytase) or non-microwave treated (BD_{UTW}; 623 U/kg of phytase) wheat meal from d 16 to 23 of age. Diets were used without or with supplementation of a phytase, alone or in combination with a xylanase. Interactions between microwave treatment and enzyme supplementation were found for $InsP_6$ hydrolysis in the ileum and P net absorption in the duodenum/jejunum and ileum. In the ileum, P net absorption was similar, but $InsP_6$ hydrolysis was significantly higher for BD_{TW} (78%) than for BD_{UTW} (69%) in the absence of supplemental phytase. Microwaving may have disrupted wheat aleurone structures in ways that increased the accessibility of the phytate and may have encouraged higher levels of activity among specific phytases of microbial or endogenous mucosal origin in the lower small intestine. In both segments, $InsP_6$ hydrolysis and P net absorption were significantly increased by supplementation of phytase, but no further by additional supplementation of xylanase. In birds that were fed the phytase-supplemented diets, microwave treatment of wheat had no effect on $InsP_6$ hydrolysis, but it significantly reduced P net absorption in both segments.

The fifth study compromised two experiments (Manuscript 5) in which the influence of different dietary factors on $InsP_6$ degradation in the crop was investigated. The experimental designs was as mentioned for Manuscript 3 (experiment 2) and 4 (experiment 1) since the samples were taken in the same trials. In experiment 1, $InsP_6$ hydrolysis in the crop was significantly increased by supplementation of phytase, but not further by the additional supplementation of xylanase. Microwave treatment of wheat reduced $InsP_6$ hydrolysis and degradation of $InsP_5$, due to reduction in intrinsic enzyme activity. The effect of 500 FTU/kg of supplemental phytase on $InsP_6$ hydrolysis was much higher in broilers fed the maize- compared to those fed the wheat-based diets (experiment 2 and 1). Thus, for supplemental phytase the accessibility of phytate in wheat seems to be lower than in maize, perhaps due to different storage sites. Supplementation of 12,500 FTU/kg of phytase caused high $InsP_6$ hydrolysis (up to 80%) and stronger degradation of $InsP_{3\text{-}5}$ than supplementation of 500 FTU/kg (experiment 2). In both experiments, degradation of $Ins(1,2,5,6)P_4$ was a limiting step in the breakdown process of $InsP_6$ by the supplemented phytase. However, upon phytase supplementation $Ins(1,2,5,6)P_4$ accumulated in BD_{TW} diets whereas $InsP_4$ degradation proceeded in untreated wheat diets (experiment 1). $Ins(1,2,5,6)P_4$ seemed to be degraded synergistically by intrinsic wheat phosphatases and the supplemented phytase.

Taking all studies together, it can be concluded that broilers and their gut microbiota have a very high potential to hydrolyze $InsP_6$ in the digestive tract when diets low in P and Ca are fed. Differences in the concentrations of lower InsPs showed that the initial step of $InsP_6$ hydrolysis is not the only catabolic step influenced by different dietary factors. To optimize efficacy of phytases and achieve a maximal InsP degradation and minimal P excretions the separate and interactive effects of different dietary influencing factors on InsP hydrolysis need to be better understood and considered in future diet formulations.

CHAPTER 6

ZUSAMMENFASSUNG

6 ZUSAMMENFASSUNG

In pflanzlichen Samen liegt Phosphor (P) überwiegend in Form von Phytat (Salze von *Myo*-Inositol 1,2,3,4,5,6-Hexakisdihydrogenphosphat oder $InsP_6$) vor. Daher hängt die P-Verfügbarkeit aus pflanzlichen Futtermitteln, die aus Samen hergestellt werden, im Verdauungstrakt hauptsächlich von der enzymatischen Hydrolyse von $InsP_6$ und von niederen Inositolphosphat-Isomeren (InsPs) ab. Aufgrund der hohen Preise für mineralischen P, der ökologischen Relevanz übermäßiger P-Ausscheidungen sowie der Erschöpfung mineralischer P-Reserven gewinnen Erkenntnisse zur Maximierung der Verwertbarkeit von Phytat-P in der Geflügelernährung zunehmend an Bedeutung. Das Hauptziel dieser Arbeit war daher, die $InsP_6$-Hydrolyse und die Bildung niederer InsPs in verschiedenen Abschnitten des Verdauungstraktes beim Broiler besser zu verstehen. Zudem wurde der Einfluss verschiedener Fütterungsfaktoren und deren Interaktionen untersucht.

In der ersten Studie (Manuskript 1) wurden P-arme (5.2 g P/kg TM), auf Mais und Sojaextraktionsschrot basierende Rationen ohne (Basalration) oder mit Zulage einer von drei Phytasen (eine *Aspergillus*- und zwei *E. coli*-Phytasen) (16.-25. Lebenstag, n=10 Abteile pro Behandlung mit je 15 Broilern) eingesetzt. Die hohe $InsP_6$-Hydrolyse (74%) bis zum Ende des Ileums der mit der Basalration gefütterten Tiere deutete auf ein hohes Potenzial des Broilers und dessen Mikrobiota zur $InsP_6$-Hydrolyse hin. Unterschiedliche InsP-Muster zwischen den Abschnitten des Verdauungstraktes lassen auf die Beteiligung von Phosphatasen mukosaler und mikrobieller Herkunft schließen. Die Phytasezulage erhöhte die $InsP_6$-Hydrolyse im Kropf, jedoch nicht im Ileum. Die Ergebnisse im Kropf und Drüsen-/Muskelmagen bestätigten die in der Literatur beschriebenen *in vitro*-Abbauwege von 3- und 6-Phytasen zum ersten Mal im Broiler. Im Dünndarm konnten die von den zugelegten Phytasen spezifisch gebildeten $InsP_{4\text{-}5}$-Isomere noch vorgefunden werden, was auf ihre weitere Aktivität im Dünndarm schließen lässt. Die Akkumulation von $InsP_4$ in den vorderen Abschnitten des Verdauungstraktes unterschied sich beim Einsatz von 3- und 6-Phytasen.

In der zweiten Studie (Manuskript 2) wurde der Einfluss der Zulage von mineralischem P und von unterschiedlichen Basalrationen auf die Verfügbarkeit von mineralischem P bestimmt. Es wurden semisynthetische oder auf Mais und Sojaextraktionsschrot (Experiment 1) und auf Mais oder Weizen basierende Rationen (Experiment 2) eingesetzt. Um P-Konzentrationen von 0,05, 0,10 und 0,15% (Experiment 1) oder 0,075% und 0,15% (Experiment 2) zu erreichen wurden die Rationen mit wasserfreiem Mononatriumphoshat (MSP_a) oder Monocalciumphosphat-Monohydrat (MCP_h) ergänzt. Experiment 1 basierte auf Exkrementsammlungen (20. bis 24. Lebenstag, n=7 Tiere pro Ration), Experiment 2 auf

Chymussammlungen (Ileum, 22 Tage alte Broiler, n=5 Abteile mit jeweils 19 Tieren). Die Weizen- und Mais-basierten Rationen unterschieden sich nicht in der $InsP_6$-Hydrolyse. Jedoch reduzierte die Zulage von mineralischem P die $InsP_6$-Hydrolyse beim Einsatz der InsP-haltigen Rationen. Die Evaluierung der zugelegten mineralischen P-Quelle wurde demnach nicht von der Wahl der Basalration beeinflusst. Allerdings sollten die für mineralische P-Quellen kalkulierten Werte um die durch die P-Zulage bewirkte Reduktion der InsP-Hydrolyse korrigiert werden.

In der dritten Studie (Manuskript 3) wurden zwei P-arme auf Mais und Sojaextraktionsschrot basierende Rationen ohne (BD-; 4.4 g P/kg TM) oder mit Monocalciumphosphat (MCP) (BD+; 5.2 g P/kg TM) ohne oder mit Zulage von 500 oder 12.500 FTU/kg Phytase eingesetzt (15.-24. Lebenstag, 20 Broiler pro Abteil, n=8 Abteile pro Behandlung). Es wurden Chymusproben aus dem Duodenum/Jejunum und Ileum entnommen. Um die Wirkung zugelegter Phytase auf die Tibia-Mineralisierung zu untersuchen, wurden weitere 180 Broiler (n=6 Abteile pro Behandlung mit je 10 Tieren, 1.-21. Lebenstag) mit den drei BD+ Rationen gefüttert. Es traten Interaktionen zwischen MCP und Phytase bezüglich der $InsP_6$-Hydrolyse sowie den Konzentrationen spezifischer niederer InsPs auf. Die Zulage von 12.500 FTU/kg Phytase ermöglichte eine $InsP_6$-Hydrolyse von 92% sowie einen starken Abbau von $InsP_5$. Zudem war die P-Nettoabsorption mit Zulage von 12.500 FTU/kg Phytase signifikant höher als ohne oder mit Zulage von 500 FTU/kg. Dies wurde durch eine höhere Lebendmassezunahme sowie durch eine höhere Tibia-Mineralisierung bestätigt. Unter der Verwendung der Rationen ohne oder mit 500 FTU/kg Phytase bewirkte die Zulage von MCP eine Reduktion der $InsP_6$-Hydrolyse sowie einen reduzierten Abbau spezifischer niederer InsPs. Beim Einsatz von 12.500 FTU/kg Phytase hatte die Zulage von MCP keinen Einfluss auf die $InsP_6$-Hydrolyse oder den Abbau von $InsP_5$. Der Einfluss von zugelegtem MCP auf die Phytaseeffizienz hängt somit von der Phytasedosis ab.

In der vierten Studie (Manuskript 4) wurden auf Weizen und Sojaextraktionsschrot basierende P-arme (4.8 g/kg TM) Rationen, welche entweder mikrowellenbehandelten (BD_{TW}; 121 U/kg Phytase) oder nicht mikrowellenbehandelten Weizenschrot (BD_{UTW}; 623 U/kg Phytase) enthielten, eingesetzt. Die Rationen wurden ohne oder mit Zulage einer Phytase allein oder in Kombination mit Xylanase verwendet (16.-23. Lebenstag, 15 Broiler pro Abteil, n=8 Abteile pro Behandlung). Zwischen der Mikrowellenbehandlung und der Enzymzulage traten Interaktionen bezüglich der $InsP_6$-Hydrolyse im Ileum und der P-Nettoabsorption im Duodenum/Jejunum und Ileum auf. Ohne Enzymzulage war die $InsP_6$-Hydrolyse im Ileum signifikant höher für BD_{TW} (78%) verglichen mit BD_{UTW} (69%), wohingegen die P-Nettoabsorption gleich war. Es ist anzunehmen, dass durch die Mikrowellenbehandlung

Strukturen in der Aleuronschicht des Weizens zerstört wurden, was die Zugänglichkeit des darin vorkommenden Phytates erhöht haben könnte. Durch die Mikrowellenbehandlung könnte die Aktivität spezifischer mikrobieller und endogener Mukosaphytasen im Darm angestiegen sein. Die Phytasezulage erhöhte die $InsP_6$-Hydrolyse und P-Nettoabsorption in beiden Abschnitten. Die Kombination mit Xylanase erzielte keine weitere Steigerung. Mit Enzymzulage wurde durch die Mikrowellenbehandlung die P-Nettoabsorption, nicht aber die $InsP_6$-Hydrolyse, reduziert.

Die letzte Studie umfasste zwei Experimente (Manuskript 5), die den Einfluss von Fütterungsfaktoren auf den $InsP_6$-Abbau im Kropf untersuchten. Das Versuchsdesign entsprach dem in Manuskript 3 (Experiment 2) und 4 (Experiment 1) beschriebenen Design. In Experiment 1 erhöhte die Phytasezulage die $InsP_6$-Hydrolyse. Die Kombination mit Xylanase erzielte keine weitere Steigerung. Infolge der reduzierten intrinsischen Phytaseaktivität wurden die $InsP_6$-Hydrolyse und der Abbau von $InsP_5$ durch die Mikrowellenbehandlung des Weizens vermindert. Die Steigerung der $InsP_6$-Hydrolyse, die durch die Zulage von 500 FTU/kg Phytase erreicht wurde, war weitaus höher unter Verwendung der Mais- (Experiment 2) als unter Verwendung der Weizen-basierten Ration (Experiment 1). Demnach scheint das Phytat in Mais für die zugelegte Phytase besser zugänglich zu sein als das Phytat in Weizen, was auf die unterschiedlichen Speicherorte zurückzuführen sein kann. Mit Zulage von 12.500 FTU/kg Phytase wurde eine sehr hohe $InsP_6$-Hydrolyse (bis zu 80%) sowie ein stärkerer Abbau von $InsP_{3\text{-}5}$ als mit der Zulage von 500 FTU/kg Phytase erreicht (Experiment 2). Beide Experimente zeigten, dass der Abbau von $Ins(1,2,5,6)P_4$ einen limitierenden Schritt beim $InsP_6$-Abbau durch die zugelegte Phytase darstellt. Jedoch scheinen die zugelegte Phytase und Phosphatasen aus Weizen $Ins(1,2,5,6)P_4$ synergistisch abzubauen, da die Phytasezulage eine Akkumulation von $Ins(1,2,5,6)P_4$ bei BD_{TW}, hingegen nicht bei BD_{UTW} bewirkte (Experiment 1).

Aus den Ergebnissen der vorliegenden Arbeit lässt sich schlussfolgern, dass Masthühner zusammen mit ihrer Mikrobiota bei Verwendung von P- und Ca-armen Rationen ein hohes Potenzial zur $InsP_6$-Hydrolyse im Dünndarm aufweisen. Unterschiede in den Konzentrationen niederer InsPs zeigten, dass die Abspaltung der ersten Phosphatgruppe beim $InsP_6$-Abbau im Verdauungstrakt nicht der einzige katabole Schritt ist, der von verschiedenen Fütterungsfaktoren und deren Interaktionen beeinflusst wird. Um die Effizienz von Phytasen zu optimieren, einen maximalen InsP-Abbau zur erreichen, den P-Bedarf des Tieres exakter zu treffen und die P-Ausscheidungen zu minimieren müssen in zukünftigen Rationsgestaltungen der Einfluss dieser Faktoren sowie deren Interaktionen auf die InsP-Hydrolyse berücksichtigt werden.

ANNEX 1. Analysed concentrations of acid detergent fibre (ADF) in feed, litter, wood shavings and content of the crop and gizzard and calculated percentage of wood shavings, litter and excreta in crop and gizzard content[1]

	Treatment	Analysed concentration of ADF (g/kg DM)	Calculated concentration of ADF in crop/gizzard content not originating from feed (g/kg DM)[2]	Calculated percentage of wood shavings in litter (%)[3]	Calculated percentage of wood shavings in crop/ gizzard content (%)[4]	Calculated percentage of litter in crop/ gizzard content (%)[5]	Calculated percentage of excreta in crop/ gizzard content (%)[6]
Feed							
	BD_{UTW}	35					
	BD_{TW}	33					
Litter							
	BD_{UTW}	479		58			
	BD_{UTW}+Phy	468		57			
	BD_{UTW}+PhyXyl	520		63			
	BD_{TW}	464		57			
	BD_{TW}+Phy	541		66			
	BD_{TW}+PhyXyl	534		65			
Wood shavings		823					
Crop content							
	BD_{UTW}	65	30		4	6	3
	BD_{UTW}	73	38		5	8	3
	BD_{UTW}+Phy	58	23		3	5	2
	BD_{UTW}+Phy	44	8		1	2	1
	BD_{UTW}+PhyXyl	52	17		2	3	2
	BD_{UTW}+PhyXyl	56	21		3	4	2
	BD_{TW}	91	58		7	13	5

ANNEX 1 continued

	Treatment	Analysed concentration of ADF (g/kg DM)	Calculated concentration of ADF in crop/gizzard content not originating from feed (g/kg DM)[2]	Calculated percentage of wood shavings in litter (%)[3]	Calculated percentage of wood shavings in crop/ gizzard content (%)[4]	Calculated percentage of litter in crop/ gizzard content (%)[5]	Calculated percentage of excreta in crop/ gizzard content (%)[6]
Crop content	BD_{TW}	79	47		6	10	4
	BD_{TW}+Phy	46	13		2	3	1
	BD_{TW}+Phy	64	31		4	6	2
	BD_{TW}+PhyXyl	73	41		5	8	4
	BD_{TW}+PhyXyl	60	27		3	5	2
Gizzard content							
	BD_{UTW}	400	365		44	76	32
	BD_{UTW}	359	324		39	68	28
	BD_{UTW}+Phy	416	381		46	81	35
	BD_{UTW}+Phy	421	386		47	82	35
	BD_{UTW}+PhyXyl	372	337		41	65	31
	BD_{UTW}+PhyXyl	436	401		49	77	37
	BD_{TW}	421	388		47	84	36
	BD_{TW}	426	394		48	85	37
	BD_{TW}+Phy	363	330		40	61	21
	BD_{TW}+Phy	476	443		54	82	28
	BD_{TW}+PhyXyl	392	359		44	67	31
	BD_{TW}+PhyXyl	422	389		47	73	34

[1]Samples of the crop and gizzard content were taken from two pens per treatment with 15 birds per pen. ADF, acid detergent fibre; Samples of the crop and gizzard content and litter samples were taken on the same day. [2]Analysed concentration of ADF in the crop or gizzard content (g/kg DM) – analysed concentration of ADF in feed (g/kg DM). Under the assumption that ADF originating from feed was not degraded in the crop and gizzard. [3]Analysed concentration of ADF in litter (g/kg DM) / analysed concentration

of ADF in wood shavings (g/kg DM) × 100. [4]Calculated concentration of ADF in the crop or gizzard content not originating from feed (g/kg DM) / analysed concentration of ADF in wood shavings (g/kg DM) × 100. [5]Calculated concentration of ADF in the crop or gizzard content not originating from feed (g/kg DM) / analysed concentration of ADF in litter (g/kg DM) × 100. Under the assumption that birds did not select between wood shavings and excreta. [6]Calculated percentage of litter in the crop or gizzard content / 100 × (100 - calculated percentage of wood shavings in litter). Under the assumption that birds did not select between wood shavings and excreta. BD_{UTW}, basal diet containing untreated wheat; BD_{TW}, basal diet containing microwave treated wheat; Phy, *E. coli*-derived phytase Quantum™ Blue; Xyl, Econase® XT 25.

Results shown in Annex 1, in Manuscript 4 and Manuscript 5 (experiment 1) are based on the same trial, respectively.

ANNEX 2. Concentrations of different InsPs in the digesta of the gizzard[1]

	BD_{UTW}			BD_{TW}			Pooled	P-value		
Supplements	0	Phy	PhyXyl	0	Phy	PhyXyl	SEM	Mwt	Enz	Mwt x Enz
$InsP_3$[2,3]	n.d.	550	391	n.d.	682	530	73	0.102	0.039	0.965
$Ins(1,2,5,6)P_4$[4]	489	2,244	1,865	n.d.	3,143	2,321	285	0.034	<0.001	0.429
$Ins(1,2,3,4,5)P_5$	424	n.d.	n.d.	342	119	n.d.	84	0.389	<0.001	.
$Ins(1,2,4,5,6)P_5$	170	n.d.	n.d.	204	n.d.	n.d.	10	0.006	.	.
$\Sigma InsP_5$	595	n.d.	n.d.	546	119	n.d.	85	0.695	<0.001	.
$InsP_6$[5]	4,136	341	253	4,479	362	329	238	0.425	<0.001	0.921

[1]Data are given as means and pooled SEM (untransformed data), *n*= 8 pens per treatment with 15 birds per pen. [2]At least one out of the following $InsP_3$ isomers: $Ins(1,4,5)P_3$, $Ins(1,2,6)P_3$, $Ins(2,4,5)P_3$, $Ins(1,3,4)P_3$, $Ins(1,4,6)P_3$. [3]Means were significantly different between Phy and PhyXyl (calculated across basal diets due to missing interactions), P≤0.05. [4]Means were significantly different all three Phy levels (calculated across basal diets due to missing interactions), P≤0.05. [5]Means were significantly different between 0 and Phy and between 0 and PhyXyl (calculated across basal diets due to missing interactions), P≤0.05. BD_{UTW}, basal diet containing untreated wheat; BD_{TW}, basal diet containing microwave treated wheat; Enz, enzyme supplementation; LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples); Mwt, microwave treatment; n.d., not detectable (the InsP isomer was not detectable in the majority of samples); Phy, *E. coli*-derived phytase Quantum™ Blue; Xyl, Econase® XT 25.

Results shown in Annex 2, in Manuscript 4 and Manuscript 5 (experiment 1) are based on the same trial, respectively.

ANNEX 3. Concentrations of different InsPs in the digesta of the gizzard[1]

		BD-			BD+			Pooled	P-value		
	Phy	0	500	12,500	0	500	12,500	SEM	Phy	MCP	Phy x MCP
$InsP_3$[2]		n.d.	518	n.d.	n.d.	845	n.d.	125	.	0.087	.
$Ins(1,2,5,6)P_4$[3]		n.d.	1,240	n.d.	n.d.	2,230	n.d.	412	.	0.114	.
$Ins(1,2,3,4,6)P_5$		65	n.d.	n.d.	78	n.d.	n.d.	12	.	0.435	.
$Ins(1,2,3,4,5)P_5$		410	59	n.d.	343	n.d.	n.d.	31	<0.001	0.186	.
$Ins(1,2,4,5,6)P_5$		503	n.d.	n.d.	453	n.d.	n.d.	23	.	0.142	.
$InsP_6$[3]		7,277	222	n.d.	7,230	385	n.d.	133	<0.001	0.182	0.131

[1]Data are given as means per treatment and SEM (untransformed data), *n*= 8 pens per treatment with 15 birds per pen; [2]At least one out of the following $InsP_3$ isomers: $Ins(1,2,6)P_3$, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$; BD-, basal diet without monocalcium phosphate (MCP); BD+, basal diet containing MCP; LOQ, limit of quantification (the InsP isomer was not quantifiable in the majority of samples); n.d., not detectable (the InsP isomer was not detectable in the majority of samples); Phy, *E. coli*-derived phytase Quantum Blue®;

Results shown in Annex 3, in Manuscript 3 and Manuscript 5 (experiment 2) are based on the same trial, respectively.

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